CHARACTERIZATION OF OOMYCETE SPECIES CAUSING SOYBEAN DISEASES IN ILLINOIS AND MANAGEMENT APPLICATIONS

BY

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THESIS

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ABSTRACT

Phytophthora root and stem rot (PRR) and seedling diseases caused by oomycetes, are two of the most important yield limitations for soybean production in the US Midwest. Several species of Phytophthora and Pythium cause PRR and seedlings diseases, respectively. PRR is managed using cultivars with single resistance genes (*Rps* genes) in combination with seed treatments. In addition to *Rps* genes, cultivars with quantitative resistance or partial resistance are available for PRR management. The objective of this thesis is to develop precision management techniques for soybean diseases caused by oomycetes. Field experiments were conducted to evaluate resistant lines combined with an ethaboxam and metalaxyl seed treatment for PRR management. Experiments were conducted in three Illinois locations in 2017. Experiments were repeated in 2018 in one location in Urbana and one location in Iowa. The seed treatment protected stands in all locations in both years, but significant yield increases were only observed in high disease pressure environments. Both resistant and susceptible cultivars benefited from the seed treatment. For the second objective, soil samples and symptomatic plants were collected from 40 counties in Illinois to characterize the population of oomycetes in the state. *Pythium ultimum* var. *ultimum* (42%) was the most abundant species, followed by *Ph. sojae* (7%) and *Ph. sansomeana* (4%). In addition, ten more *Pythium* species were identified. Virulence of all *Phytophthora* spp. isolates was evaluated by inoculating 12 soybean differentials with known *Rps* genes. Sixteen pathotypes were identified among the *Ph. sojae* isolates, and no pathotypes were identified for *Ph. sansomeana*. The aggressiveness and fungicide sensitivity of the isolates was also evaluated. Aggressiveness assays were performed in the greenhouse for *Phytophtora* spp. isolates, and a petri plate assay was used to assess the aggressiveness of *Pythium* isolates. *Ph. sojae* was more aggressive compared to *Ph. sansomeana. Py. ultimun* var. *ultimum, Py. ultimun* var. sporangium, *Py. irregulare* and *Py.*

aphanidermatum were the most aggressive *Pythium* species to soybean. Both *Phytophthora* spp. were sensitive to metalaxyl, mefenoxam, azoxystrobin, and ethaboxam. There were Pythium isolates insensitive to azoxystrobin and ethaboxam.

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TABLE OF CONTENTS

CHAPTER 1: EFFECT OF RESISTANCE AND ETHABOXAM SEED TREATMENT ON THE MANAGEMENT OF PHYTOPTHORA ROOT ROT IN ILLINOIS AND IOWA[1]

ABSTRACT

Phytophthora root and stem rot (PRR) is a limiting factor for soybean production. Seed treatments are used for early season management, but efficacy can depend on seed selection and the local environment. Ethaboxam is a new fungicide commercially available as a seed treatment to control oomycetes. Field experiments were established in Illinois and Iowa in 2017 and 2018 to evaluate the effect of ethaboxam + metalaxyl on PRR. Experiments included soybean lines with no resistance gene, *Rps*1c and *Rps*1k, and different levels of partial resistance. Seed treatments increased soybean stands in all locations and years. Significant yield effects were observed only in two locations that were inoculated with *Phytophthora* spp. Groups of soybean lines with the same *Rps* gene responded differently in each location, showing how *Rps* gene usefulness depends on the field. A comparison of the effect of seed treatment on lines with different levels of partial resistance showed that partial resistance alone cannot always protect against stand losses. Soybean lines with high levels of partial resistance had consistently higher yields than those with low levels of partial resistance across Illinois locations. These results show that ethaboxam seed treatment can protect early season stands and that selection of cultivars with high levels of partial resistance is important for PRR management.

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INTRODUCTION

In the past two decades, Phytophthora root and stem rot of soybean (PRR) has consistently been classified as one of the most destructive soybean diseases in the northern United States (Allen et al. 2017; Koenning and Wrather 2010; Wrather and Koenning 2006). Phytophthora root and stem rot is mainly caused by the oomycete *Phytophthora sojae* Kaufmann & Gerdemann and at a lesser degree by *Phytophthora sansomeana* E.M. Hansen & Reeser (Malvick and Grunden 2004; Rojas et al. 2017). PRR reduces yield due to early stand loss and plant vigor reduction late in the season (Dorrance 2018). Initial symptoms of PRR include pre- and post-emergence damping-off, and rotted seeds and seedlings with brown lesions. Late season symptoms are dependent on the levels of partial resistance of the cultivar (Dorrance 2018). Cultivars with low levels of partial resistance develop root rot, brown lesions, and wilting. Symptoms in cultivars with greater levels of partial resistance are normally limited to the roots and yield loss is minimal (Schmitthenner 2000). *Phytophthora sojae* can survive for many years as oospores in the soil and crop residue and PRR is more severe in poorly drained soils (Schmitthenner 2000; Dorrance 2018). Integrated management that includes host resistance, seed treatment, and soil drainage is recommended for successful disease control (Dorrance 2018).

Cultivars with single genes for resistance to *Ph. sojae* (*Rps*) and multigenic partial resistance are available to manage this pathogen. The most commonly used *Rps* genes in commercial cultivars are *Rps*1a, *Rps*1c, and *Rps*1k (Slaminko et al. 2010) and no single *Rps* gene confers resistance to all pathotypes. In the most recent pathotype survey, more than 50% of isolates recovered from Illinois and Iowa fields were virulent to *Rps*1a, *Rps*1c, and *Rps*1k (Dorrance et al. 2016). Selection of cultivars with partial resistance (also referred to as tolerance) is essential for disease management as *Ph. sojae* populations quickly adapt to *Rps* genes (Stewart et al. 2014;

Dorrance et al. 2016). Partial resistance is effective against all pathotypes, and cultivars with partial resistance also tend to have higher yields when the disease is present late in the season (Dorrance et al. 2003; Dorrance et al. 2009). Recently, Garnica and Giesler (2019) reported in Nebraska that cultivars with moderate levels of partial resistance yielded higher than moderately susceptible cultivars, but no difference in yield was seen between cultivars with *Rps*1k and *Rps*1c. As pathotype diversity is increasing and partial resistance is not effective at planting, the use of resistant cultivars in combination with seed treatments is necessary to ensure seed protection early in the season (Dorrance and McClure 2001; Dorrance 2018).

The adoption of early planting and conservation tillage have favored early-season disease, which increased the need for seed treatment protection (Rowntree et al. 2013; Vossenkemper et al. 2015). Seed treatments can increase stand count and yields, but these positive results are dependent on factors such as the cultivar and environment (Bradley, 2007; Dorrance et al. 2009; Esker and Conley 2012; Garnica and Giesler 2019; Gaspar et al. 2014; Rossman et al. 2018). For PRR management, seed treatments have been effective when rain or irrigation occurs after planting (Dorrance et al. 2009; Garnica and Giesler 2019; Scott et al. 2020). Metalaxyl and mefenoxam have been widely used for oomycete management since their introductions (Dorrance 2018). A new compound, ethaboxam (Valent U.S.A. Corporation, Walnut Creek CA), has been recently introduced as a seed treatment for soybean and cereals. Ethaboxam is effective against varoious oomycetes species including those insensitive to metalaxyl and mefenoxam (Kim et al. 2004; Radmer et al. 2017; Rojas et al. 2019). Some species have inherent insensitivity to ethaboxam, so including ethaboxam in combination with metalaxyl or mefenoxam is recommended for protection against most oomycetes (Noel et al. 2019; Scott et al. 2020).

Due to the pathogen diversity and seed treatment effect variability across environments, recommendations for cultivar and seed treatment selection should be made on a regional basis for successful PRR management. In order to locally evaluate an ethaboxam and metalaxyl based seed treatment in combination with *Rps* genes and partial resistance as a PRR management practice, field experiments were established in multiple locations in Illinois and one location in Iowa. Soybean lines with no resistance gene, *Rps*1c or *Rps*1k and different levels of partial resistance were selected for field trials in Illinois. Commercial cultivars carrying *Rps*1c or *Rps*1k were used for a field trial in Iowa.

MATERIALS AND METHODS

Screening of resistance genes (*Rps*) in public soybean lines.

A set of 30 public soybean lines from the University of Illinois soybean breeding program were screened for resistance to *Ph. sojae* . The presence of resistance genes was determined using the hypocotyl inoculation technique (Dorrance et al. 2008). Twelve seeds per line were sown in 1.4-liter pots with coarse vermiculite. The susceptible cultivar, Sloan, with no resistance gene (*Rps*) was used as a control. Pots were kept in the greenhouse at 25°C and seedlings were inoculated one week after planting when cotyledons had expanded. Soybean lines were inoculated using isolates with the pathotypes: OHR1 (7), OHR3 (1a), OHR4 (1a, 1c), OHR7 (1a, 3a, 6), and OHR25 (1a, 1b, 1c, 1k) (Dorrance et al. 2008). All isolates were grown on Lima Bean agar (LBA: 150 g autoclaved lima beans, 20 g bacto agar per liter) for two weeks before inoculation (Dorrance et al. 2008). Inoculations were performed by making a slit in the hypocotyl and placing into it between 0.2 to 0.4 ml of macerated culture (mycelium and LBA agar). A resistant reaction was recorded when ≤ 25% of the seedlings were killed; 26 to 75% seedlings killed indicated an

intermediate reaction; and ≥ 75% seedlings killed was recorded as susceptible. Intermediate reactions can be an indication of contamination (Dorrance et al. 2008). Inoculations of each soybean line were repeated three times.

An additional growth chamber screening was conducted to confirm results of greenhouse assays. All 30 lines were inoculated with the isolate OHR1 and lines that were resistant to OHR1 were grown again and then inoculated with isolate OHR3 and then OHR4. Twelve seeds per line were placed and rolled on germination paper (Anchor Paper Co., Saint Paul, MN) and incubated at 25°C in 25-liter plastic containers. Seedlings were inoculated a week later using the hypocotyl inoculation technique described above and rated a week after inoculation. Each line by isolate combination was replicated three times in different 25-liter plastic containers. Isolates were grown on LBA for two weeks before inoculation. Soybean cultivars Harlon (*Rps*1a), Harosoy (*Rps*7), Harosoy 13XX (*Rps*1b), Haro 62XX (*Rps*6), Williams 79 (*Rps*1c), and Williams 82 (*Rps*1k) were used as controls (Dorrance et al. 2004).

Screening of partial resistance in public soybean lines

The levels of partial resistance were evaluated using the layer and the tray tests (Dorrance et al. 2008). For the layer test, polystyrene cups (946 mL) were filled with an initial layer of coarse vermiculite followed by *Ph. sojae* inoculum and then by a second layer of coarse vermiculite. Ten seeds per line were placed in the cup and covered with vermiculite. Inoculum was placed nine cm below the seeds. Due to all lines being susceptible to OHR25 when using the hypocotyl inoculation technique, this isolate was used for inoculum. The isolate was previously grown in LBA for two weeks. Cups were maintained in the greenhouse at 25°C. The experiment was arranged in a randomized complete block design with 3 replicates per line. Each cup was considered an experimental unit. Seedlings were rated three weeks after planting using a scale from 1 to 9, were

1 indicates no root rot, 3 indicates bottom third of root mass rotted and a 9 means all seedlings were dead. Lines with scores between 1.0 to 4.0 were classified as having high levels of partial resistance, lines with scores between 4.1 and 6.0 were classified as having moderate levels of partial resistance, and lines with scores over 6.0 were classified as having low levels of partial resistance.

The tray test was used to measure lesion length, one of the components of partial resistance (Mideros et al. 2007). Ten seeds per line were sown in polystyrene cups (946 ml) filled with vermiculite and kept in the greenhouse at 25°C for one week. After one-week, seedlings were removed from cups and roots were washed with tap water. Eight to ten seedlings were placed in the trays and a wound was made on the tap root. Seedlings were inoculated by placing a mycelial slurry of a two-week-old *Ph. sojae* culture on the wound. Trays were stacked together and placed in a 25-liter plastic container with two liters of water. The experiment was established in a randomized blocks design with three replicates (each block was a plastic container). Containers were incubated at 25°C with a 14-hour photoperiod. Sloan (*Rps*, low partial resistance), Williams (*Rps*, moderate partial resistance), and L76-1988 (*Rps*2) were used as controls. Lesion length was measured a week later from the inoculation site to the end of symptomatic tissue. Analysis of variance (ANOVA) of lesion length data was conducted in R version 3.6.3 using the 'aov' function (R Core Team 2020). The 'boxcox' function from the 'MASS' package was applied to lesion length (Venables and Ripley 2002). Line was considered a fixed effect. Tukey's HSD was used for mean separation using the 'emmeans' package (Lenth 2020).

Multilocation field trials in 2017

Experiments were established in Illinois in fields with seedling disease history at the Northwestern Illinois Agricultural R&D Center (Monmouth), the Orr Agricultural R&D Center (Orr), and the Crop Science Research and Education Center (Urbana). Experiments were arranged in a split-plot design with four replications in randomized complete blocks, with line as the whole plot factor and seed treatment (seed treatment and non-treated control) as the subplot factor. The fungicide and insecticide treatment, Intego Suite Soybeans (Valent U.S.A. Corporation, Walnut Creek CA), was applied to each line at the recommended rate of 3.37 fl oz per 100 lb of seed (clothianidin: 0.081 mg, ethaboxam: 0.012 mg, ipconazole: 0.004 mg, metalaxyl: 0.0032 mg of active ingredient per seed). Clothianidin is an insecticide and ipconazole is a broad-spectrum fungicide. The active ingredients target different insect pests, seedborne diseases and seedling diseases.

Planting occurred on May 18 for Urbana, May 16 for Monmouth and May 10 for Orr. Plots were four rows wide and rows were 0.76 m apart. In Urbana and Monmouth, row length was 5.1 m and seeding rate was 344,445 seeds/ha. In Orr, row length was 6.9 m and seeding rate was 433,247 seeds/ha. Stand counts were collected at VC and V2 growth stages. A final stand count at R8 was collected only in Urbana. Stand count consisted of the number of plants alive in one meter measured in each of the two central rows and transformed to plants/m^2 for analysis. Plots were harvested with a research combine and yield was adjusted to 13% moisture content for analysis.

A multilocation statistical analysis was performed in R version 3.6.3 (R Core Team 2020). A linear mixed model was used to evaluate stand counts and yield with line and seed treatment as fixed effects and whole plot error and replication nested in location as random effects. Packages ‘lme4’ and ‘lmerTest’ were used to fit the model and perform ANOVAs (Bates et al. 2015;

Kuznetsova et al. 2017). Diagnostic residual plots to check on the model assumptions were created using the package 'ggResidpanel' (Goode and Rey 2019). The significance level was set to $\alpha < 0.05$. Estimated marginal means and contrasts were performed using the 'emmeans' package using the multivariate-t adjustment (Lenth 2020). Contrasts between the non-treated control and seed treatment were performed to evaluate the response to seed treatment by type of resistance. For this analysis, lines were grouped by resistance gene (*Rps*, *Rps*1c, *Rps*1k) or by levels of partial resistance (high, moderate, low). Because few of the yield contrasts by level of partial resistance found significant effects for the seed treatment, we also conducted a contrast analysis by levels of partial resistance on the combined treated and not treated data. Figures were created using 'ggplot2' (Wickham 2016). Data and code used for analysis can be found at https://github.com/danielcerritos/seedtreatments.

Urbana and Boone field trials in 2018

In 2018, field experiments were established in the Crop Science Research and Education Center near Urbana, Illinois and at the Field Extension and Education Laboratory in Boone, Iowa. Experiments were arranged as a split-plot design with Intego Suite Soybeans or a non-treated control applied to the subplots as indicated above. The seed treatment was applied at the same rate as for the 2017 field trials. The Urbana trial in 2018 had six lines in common with 2017 and extra lines were added to include more low partial resistance options in the 2018 trials. In Iowa, commercial cultivars were selected for the experiment. Planting occurred on April 30 for Urbana at a rate of 308,882 seeds/ha and June 5 for Iowa at 296,526 seeds/ha. Plots were four rows wide (0.76 m row spacing) with a row length of 5.3 m for both locations.

The Urbana field was irrigated after planting using impact sprinklers (Rain Bird, Azusa, CA) for five days after planting to add an extra 127 mm of water. This field experiment was also

inoculated with 6.6 g/m of *Ph. sojae* and *Ph. sansomeana* colonized millet applied in furrow at planting. To produce inoculum, white millet grain was washed three times then soaked overnight in buckets filled with water. The next day water was drained, and millet was rinsed three times. The millet was placed in autoclave bags (Fisherbrand) and autoclaved for an hour. Twenty-four hours later, grain was autoclaved a second time. Cooled grain was inoculated with *Phytophthora* spp. culture by adding two plates of macerated culture and 30 ml of autoclaved water to each bag. Isolates 17PR018J.3 (*Ph. sojae* : 1b, 1k, 5, 7) and 16PR018B.1 (*Ph. sansomeana*), collected in Boone and Kankakee counties in Illinois, respectively, were used for inoculum. Isolates were grown at 25°C for two weeks on LBA. Grain and *Phytophthora* spp. culture were mixed and then the bags were incubated in the dark for two weeks. The inoculum was mixed every three days. After two weeks, the inoculum was dried for 12 hours at 38°C in a forced air oven and then ground using a mill (Bell Co., Tiffin, OH). Inoculum was placed in 50 lb brown SOM Bags (MIDCO Global, Kirkwood, MO) and stored in a cold room until planting.

The trial in Iowa was inoculated with 9.4 g/m of *Ph. sojae* - colonized millet applied in furrow at planting. Briefly, white grain millet was soaked for 24 hours in deionized water and then drained. Approximately, 500 ml of millet was placed in a spawn bag (0.2-micron pore patch) (MycoSupply Company, Pittsburgh, PA) and autoclaved for 30 minutes. After 24 hours, bags were autoclaved again for 30 minutes. Twenty-four hours later, a Petri dish (100 mm x 15 mm) colonized with a *Ph. sojae* isolate was added to each bag. Two isolates were used; 1005-2.9 (1a, 1b, 1c, 1k, 3b, 7), which was recovered in 2009 from a soil sample (Stewart et al. 2014), and IA22-7-1 (1a, 1b, 1c, 1k, 3a, 3b, 3c, 7, which was isolated from a symptomatic seedling in 2012 (Dorrance et al. 2016). Isolates were grown at 23°C for 7 to 10 days on dilute V-8 juice agar (Dorrance et al. 2008) amended with the two antibiotics (neomycin sulfate (100 mg/liter), and

chloramphenicol (10 mg/liter)). Bags were incubated at 23°C for 10 to 14 days with occasional mixing of the bags to ensure colonization of each millet grain. Inoculum was then dried at 23°C in a fume hood for 2 days.

Data collection and analysis of field trials in 2018

Three-meter segments were flagged in the two central rows of each plot to collect stand count data. The number of plants alive in the flagged segments were counted at VC, V2, V4, and R8 in the Urbana experiment and VC, V1, R2, and R8 growth stages in the Iowa experiment. Plots were harvested in October and yield was adjusted to 13% moisture content. Stand counts were transformed to plants/m^2 for analysis. Statistical analysis was as described above for the 2017 field experiment. Urbana 2018 and Boone data were analyzed separately.

RESULTS

Screening results and cultivar selection for field trials

Twenty of the public soybean lines did not have any resistance gene, six lines had *Rps*1c, three had *Rps*1k and one had *Rps*1a (Supplemental Table 1). Mean scores from the layer test ranged from 3.17 to 8.0. Based on the layer test scores, six lines were classified as having high levels of partial resistance, 12 were classified as moderate partial resistance, and 12 were classified as low partial resistance (Supplemental Table 1). The mean lesion lengths were 18.2 mm, 18.6 mm, and 20.4 mm for lines classified as high, medium, and low partial resistance in the layer test, respectively. Tukey's HSD test on the lesion lengths showed significant differences between lines with high and low partial resistance, but most lines classified as moderate where not significantly different from either high or low.

Seven lines carrying *Rps*1c, *Rps*1k, or no major resistance gene (*Rps*) were selected for multilocation field trials in 2017 (Table 1). Lines with *Rps*1c or *Rps*1k were selected because these genes are the most used genes in soybean production (Slaminko et al. 2010). Another factor for the selection of soybean lines was to include various levels of partial resistance. For the 2018 field trial in Urbana, we selected six lines from the 2017 field trials and three additional lines with low levels of partial resistance.

Commercial cultivars with either *Rps*1c or *Rps*1k were use in the field trial in Iowa. Tolerance scores on a scale from 1 to 9 from the seed companies were used for classification. The Asgrow (AG) cultivar had a score of 6 and the Golden Harvest (NK) cultivars had scores of 3 and 4. For Asgrow and Golden Harvest scale, 9 means low resistance. Hoegemeyer (H) cultivars had scores of 4 and 5, and LG seeds (C) cultivars had scores of 7 and 9. For Hoegemeyer and LG seeds, 9 means high tolerance. For this study we translated this information into levels of partial resistance (Table 1).

Multilocation field trials in Illinois 2017

The seed treatment significantly improved soybean stands at both VC and V2 ($P < 0.001$). The seed treatment increased stand counts by 12.8% at VC and 8.2% at V2. The location by seed treatment and line by seed treatment interactions were non-significant. Seed treatment was not significant for yield ($P = 0.49$) although greater yield (0.6%) was seen across locations for the plots with the seed treatment compared to the non-treated plots. The location effect for stand count at both growth stages and for yield was significant ($P < 0.001$). The location by line interaction was significant for stand counts at VC ($P = 0.004$) and for yield ($P < 0.001$). To investigate if some locations favored lines with specific types of resistance, an analysis by location was also performed.

Monmouth 2017. As observed in the multilocation analysis, seed treatment was a significant factor for stand counts at VC (P = 0.001) and V2 (P = 0.001), but not for yield (P = 0.95). Line was also a significant factor for stand counts at VC (P = 0.03) and for yield (P = 0.006). The interaction between line and seed treatment was not significant. The contrasts by resistance gene group showed a significantly higher stand (19.6%, P = 0.009) at VC for the treated *Rps*1c lines compared to the non-treated, but the effects were not significant for *rps* or *Rps*1k lines (Figure 1; Supplemental Table 2). When grouped by levels of partial resistance, no significant differences in stand were observed between seed treated and the non-treated control for any of the partial resistance groups. When the analysis was conducted by line, seed treatment significantly increased stand (25%) only for LD11-10069 (*Rps*1c, moderate PR) compared to the non-treated control (Figure 1). In the yield contrasts of the combined dataset (treated and non-treated), lines with high partial resistance yielded significantly more (13%, P = 0.04) than the line with low partial resistance (Figure 2; Supplementary Table 3).

Orr 2017. Seed treatment was a significant factor at both VC (P < 0.001) and V2 (P = 0.02), but not for yield (P = 0.43). Line was a significant factor at VC (P = 0.001) and yield (P < 0.001). The line by seed treatment interaction was not significant. The contrasts by resistance gene group showed a significantly greater stand (30%, P = 0.03) at VC for the treated *Rps*1k lines but these contrasts were not significant for *rps* or *Rps*1c lines (Figure 1; Supplementary Table 2). The seed treatment significantly increased the stand for the lines with moderate (18.7%, P = 0.03) and low levels (41%, P = 0.03) of partial resistance but had no significant effect on the lines with high levels of partial resistance (Supplementary Table 2). At this location, in the analysis by line, seed treatment significantly increased stand only for LD12-15156R1a (*Rps*1c, moderate PR) from 17.8 plants/m^2 to 24.5 plants/m^2 (Figure 1). In the yield contrasts with the combined treated and non-

treated data, high partial resistance lines yielded 221 kg/ha (4.3%, $P = 0.006$) more than moderate and 555 kg/ha (11.5%, $P < 0.001$) more that the line with low partial resistance (Figure 2; Supplementary Table 3).

Urbana 2017. Seed treatment was a significant factor for stand counts at VC ($P < 0.001$), V2 ($P = 0.02$), and R8 ($P < 0.001$), but not for yield ($P = 0.56$). Line affected stand at VC ($P = 0.005$) and yield ($P < 0.001$). The line by seed treatment interaction was significant at VC ($P = 0.01$). Response to seed treatment varied by resistance gene. A significant increase in stand for seed treatment was observed for lines with *Rps*1k (19.1%, $P < 0.001$) and *rps* (14.3%, $P < 0.001$), but not for lines with *Rps*1c (8%) (Figure 1, Supplementary Table 2). The seed treatment significantly increased the stand for lines with all levels of partial resistance (8.5% for high PR, 12.3% for moderate PR, and 12% for low PR) (Supplementary Table 2). In the analysis by soybean line, seed treatment significantly increased the stand count of all lines with no resistance gene and *Rps*1k but stand increased for only one (LD12-15156R1a) of the three *Rps*1c lines (Figure 1). In the yield contrasts with the combined treated and non-treated data, high and moderate partial resistance lines yielded 628 kg/ha (13.3%, $P = 0.001$) and 586 kg/ha (12.4%, $P < 0.001$) more than the low partial resistance line (Figure 2; Supplemental Table 3).

Urbana and Boone field trials in 2018

Urbana 2018. Seed treatment plots had higher stands at VC (17.8%, $P < 0.001$), V2 (18.6%, $P < 0.001$), V4 (18.4%, $P < 0.001$), and R8 (15.6%, $P < 0.001$) compared to the non-treated control. Yields were also significantly higher ($P < 0.001$) for the seed treated plots (3364 kg/ha) than for the non-treated control (3122 kg/ha). The seed treatment increased yield by 7.8% across lines. The line affected stand at VC ($P = 0.04$) and for yield ($P < 0.001$). The line by seed treatment interaction was non-significant for all stand counts and yield. The seed treatment

contrasts by resistance gene group showed a significantly increased stand for treated compared to non-treated plots for lines with *Rps*1k (26.6%) and for those with no resistance gene (24.5%) (Figure 3A; Supplemental Table 2). No difference between the non-treated control and seed treatment was observed for *Rps*1c lines (Supplemental Table 2). When grouped by levels of partial resistance, there was a significant difference ($P = 0.015$) between treated and non-treated plots for the lines with moderate levels of partial resistance. When the response was analyzed by line, seed treatment significantly increased stands of two lines (LD07-3395bf and LD11-7311) by 48% and 31.4%, respectively (Figure 3A). Seed treatment also increased yields of the lines LD07-3395bf and LD13-14071R2 by 17.36% and 10.7%, respectively. In the yield contrasts by partial resistance group with the combined treated and non-treated data, lines with high levels of partial resistance yielded 830 kg/ha (27.7%, $P < 0.001$) and 648 kg/ha (20.4%, $P < 0.001$) more than lines with low and moderate levels of partial resistance (Figure 3B Supplemental Table 3).

Boone 2018. In Iowa, seed treatment plots had significantly higher stands at VC (12.3%, $P = 0.008$), V1 (9.6%, $P = 0.04$), R2 (11.1%, $P = 0.009$), and R8 (12%, $P = 0.006$) compared to the non-treated controls. Yields were significantly higher ($P = 0.007$) for the seed treatment (2987 kg/ha) than for the non-treated control (2763 kg/ha) with an increase of 8.1% across cultivars. In the ANOVA, cultivar was not a significant factor for stand count and for yield. The cultivar by seed treatment interaction was non-significant for stand count and yield. Response to seed treatment varied by resistance gene group. The cultivars with *Rps*1c and the seed treatment had a significant increase in both stand at VC (15.5%, $P = 0.009$) and yield (7.8%, $P = 0.04$) compared to the non-treated control (Figure 4A; Supplemental Table 2). No difference between the non-treated control and the seed treatment was observed for cultivars with *Rps*1k for both stand at VC and yield. When grouped by levels of partial resistance, there was a significant difference ($P =$

0.029) between treated and non-treated plots for the lines with high levels of partial resistance for stand count at VC. In the analysis by cultivar, only C3140RX (*Rps*1c, high PR) showed significant differences between the non-treated control and seed treatment for both stand at VC (34%, Figure 4A) and yield (20%). In the yield contrasts by partial resistance group on combined treated and non-treated data, there was no significant difference between high and moderate levels of partial resistance (Figure 4B; Supplemental Table 3).

DISCUSSION

Host resistance and seed treatments are available for effective management of PRR, but these strategies do not offer foolproof control of the disease. The pathogen can overcome host resistance, and seed treatments are useful only for a few weeks after planting. *Phytophthora* sojae pathotype diversity and complexity have increased, and each state and field may have distinct populations (Dorrance et al. 2016; Stewart et al. 2015). The most commonly grown soybean cultivars have *Rps*1k, *Rps*1c, or *Rps*1a (Slaminko et al. 2010), and it is essential to evaluate if these genes are still useful for *Ph. sojae* management at the local level. New active ingredients that target oomycete pathogens (e.g., ethaboxam and oxathiapiprolin) have recently been introduced for seed treatment. Field trials are needed to evaluate the efficacy of both management strategies. This two-year field study was established in four environments in Illinois and one in Iowa to evaluate the efficacy of ethaboxam + metalaxyl as a seed treatment combined with resistant cultivars for *Ph. sojae* management.

The seed treatment increased early stand counts (VC-V2) in all five environments. Seed treatment efficacy can depend on environmental conditions, so recommendations can be more effective when made to a specific region or state (Dorrance et al. 2009; Gaspar et al. 2014;

Rossman et al. 2018). In Ohio, ethaboxam + metalaxyl improved early-season stands across various fields with seedling disease history (Scott et al. 2020). In contrast, Garnica and Giesler (2019) reported that ethaboxam + metalaxyl did not significantly increase stand counts across different environments with PRR history in Nebraska. The cultivars used at the Iowa location were identical to those used in the Nebraska studies in 2018 (Garnica and Giesler 2019). Unlike Iowa, the Nebraska studies were not inoculated. In both the Ohio and Nebraska studies, positive responses in stands were observed with treatment when fields received enough precipitation during the first two weeks after planting. In our study, environments received between 53.1 and 185.4 mm of water during the first two weeks after planting, which favored disease development. Early season stands increased in all environments with seed treatment compared to non-treated plots, but the highest responses were observed in Orr 2017 (18.0%) and Urbana 2018 (17.9%), where precipitation was 122.9 and 179.8 mm during the first two weeks. Supplemental irrigation was provided to Urbana 2018 to increase disease pressure. Boone received more than double the precipitation than the Nebraska locations received in 2018. Thus, together with the inoculum at Boone, conditions were likely more conducive for PRR at the Iowa location compared to the Nebraska test locations. Effect of early-season protection was observed season long as mid (V4-R2) to late (R8) stand counts were higher in the seed treatment plots.

The seed treatment consistently protected stands in all environments, but this protection translated into significant yield responses in just two locations. Dorrance et al. (2009) reported stand protection from *Ph. sojae* and yield increases from seed treatments in environments that received excessive precipitation or irrigation after planting. In our study, all environments received enough precipitation and disease developed based on the significant differences in early stands between the seed treatment and non-treated plots. The seed treatment significantly increased yields

in Urbana 2018 (7.8%) and Boone (8.1%). Both environments were inoculated, which probably caused higher disease pressure. Early season protection was observed in 2017 locations, but the yield was probably not influenced because stands were high enough to compensate (De Bruin and Pedersen 2008). Also, the 2017 environments had higher seeding rates compared to Urbana 2018 and Boone. Other studies had reported similar results where no significant yield response was observed under high seeding rates, although early season protection was observed (Cox and Cherney 2014; Vossenkemper et al. 2015; Rossman et al. 2018). Soybeans can compensate well when stands are reduced by producing additional yield on branches (Cox and Cherney 2011; Suhre et al. 2014).

For stand counts, contrasts between groups of soybean lines with different *Rps* genes had varied responses to seed treatment at each location. A significant seed treatment effect for a group of soybean lines with the same *Rps* gene can be interpreted as a failure by that gene in that field. We observed this effect for *Rps*1c lines in Monmouth and Boone, and *Rps*1k lines in Orr and two years in Urbana. These results suggest that soybean lines with *Rps*1k and without a seed treatment would have been an effective management strategy in Monmouth and Boone but not in Orr nor Urbana. At the same time, soybean lines with *Rps*1c would have been effective in the Orr and Urbana fields, on average. These differences are expected due to the variable presence of *Ph. sojae* pathotypes (races) between fields (Dorrance 2016; Scott et al. 2020). It also illustrates that using single genes alone to control PRR can only be effectively done if the pathogen diversity for each field is known before planting.

We also found significant seed treatment differences between lines with different levels of partial resistance for stand counts. A significant effect of the seed treatment for a group of lines with the same levels of partial resistance can be interpreted as a failure of this type of resistance.

We observed this effect on at least two locations for each of the levels of partial resistance. These results agree with previous reports that partial resistance is not effective in field trials in the first few weeks after planting (Dorrance et al. 2003; Dorrance and McClure, 2001). However, this effect was not consistent across levels of partial resistance or in all locations. It is important to note that several confounding factors are in play in these experiments, such as the fact that soybean lines also have *Rps* genes and that other oomycetes such as *Pythium* spp. are known to reduce stands under similar environmental conditions. In this study, *Pythium ultimun ultimum* var. *ultimun ultimum* was isolated from the Urbana 2018 field. This species is pathogenic on soybeans and it has been reported to be insensitive to mefenoxam (Rojas et al. 2016; Rojas et al. 2019). It is also possible that other pathogens that cause seedling diseases such as *Rhizoctonia solani* and *Fusarium* spp. contributed to stand reduction at these locations. Our study agrees with previous reports that using partial resistance alone does not always ensure early season protection. Thus, effective management would combine seed treatment and partial resistance.

As for the overall analysis, the responses for stand by *Rps* group and by partial resistance group did not necessarily translate into yield effects. Although a few yield contrasts by *Rps* or partial resistance showed significant differences between treated and non-treated plots, no clear pattern was observed (Supplementary Table 2). In addition to the limitations of our experiments indicated above, small plots tend to be underpowered to detect small yield differences (Kandel et al. 2018; Lin et al. 2020). We also looked at the contrast of partial resistance levels but with the combined treated and non-treated data.

Previous field trials have reported higher yields for lines with high partial resistance (Dorrance et al. 2003; Dorrance et al. 2009). Our data also finds significantly higher yields for soybean lines that were rated with high levels of partial resistance than those with low partial

resistance in four of the five locations. The exception was Boone where commercial cultivars were used and none of which was reported as having low levels of partial resistance. Overall, high partial resistance lines yielded, on average, 647.6 kg/ha (21.5%) and 396.5 kg/ha (13.2%) more than low and moderate partial resistance lines, respectively. Although this is an interesting result shown across several studies and locations, it is important to consider that soybean lines are different in other agronomic traits and yield differently regardless of partial resistance. Further investigations are warranted in this area.

We have shown the importance of cultivar selection combined with seed treatments for management of *Ph. sojae* in Illinois and Iowa. The seed treatment with ethaboxam + metalaxyl was shown to improve the early season stands across environments. Significant yield increases were variable but still observed in two high disease pressure locations. Yield increases were found in environments where the stand was significantly reduced early or throughout the season. The seed treatment benefited both susceptible and resistant lines under higher disease pressure. Cultivars with *Rps* genes are still valuable for disease management, but effectiveness can vary by location. No clear benefit of partial resistance was observed at the early growth stages. However, the selection of high partially resistant cultivars might produce higher yields across different environments. Planting cultivars with high levels of partial resistance combined with a seed treatment can be the most effective way of managing *Ph. sojae* and seedling diseases.

TABLE AND FIGURES

Table 1. Lines and cultivars selected for field trials

State and Year	Line or cultivar	Line abbreviation[a]	Rps	Partial Resistance[b]
Illinois 2017	LD11-10069	LD11-10	*Rps*1c	Moderate
Illinois 2017 and 2018	LD07-3395bf	LD07-33	*rps*	High
	LD10-10219	LD10-10	*rps*	Moderate
	LD11-13802R2	LD11-13	*Rps*1k	Moderate
	LD11-7311	LD11-73	*Rps*1k	Moderate
	LD12-15156R1a	LD12-151	*Rps*1c	Low
	LD13-14071R2	LD13-14	*Rps*1c	High
Illinois 2018	LD12-15129R1a	LD12-151a	*Rps*1c	Low
	LD12-15064R1a	LD12-150	*Rps*1c	Low
	LD13-13478R1a	LD13-13	*rps*	Low
Iowa 2018	AG28x7		*Rps*1c	Moderate
	H2862NX		*Rps*1k	Moderate
	H2512NX		*Rps*1k	Moderate
	NK3195X		*Rps*1c	High
	NK2788X		*Rps*1c	Moderate
	C2888RX		*Rps*1c	High
	C3140RX		*Rps*1c	High

[a] Abbreviations of lines used in Figures 1-3

[b] Partial Resistance levels for the public soybean lines were determined in this study through the layer test. For the commercial soybean lines, the values shown are a translation of the reported levels of tolerance to *Ph. sojae* .

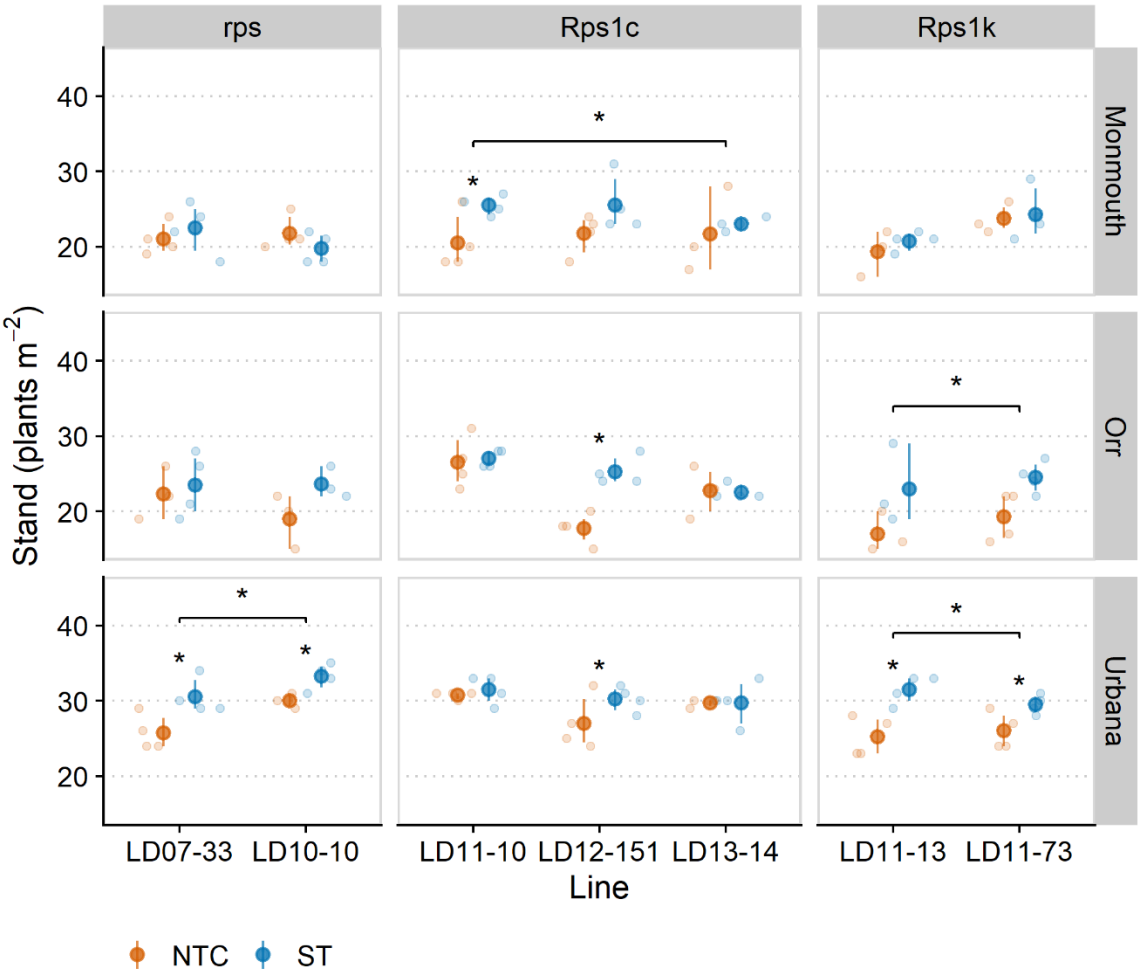

Figure 1. Effect of seed treatment and *Rps* gene on stand count (at VC) in multilocation field experiments in Illinois 2017. Dots represent the mean stand of seed treatment plots(ST - blue) or non-treated control plots (NTC - orange). Vertical bars show confidence intervals of the means. Panels with a bracket and asterisk represent a significant difference in the contrast between the non-treated control and the seed treatment when soybean lines were grouped by resistance gene. An asterisk above a soybean line represents a significant difference in the contrast between the non-treated control and the seed treatment for that soybean line only. Abbreviations of lines are defined on Table 1.

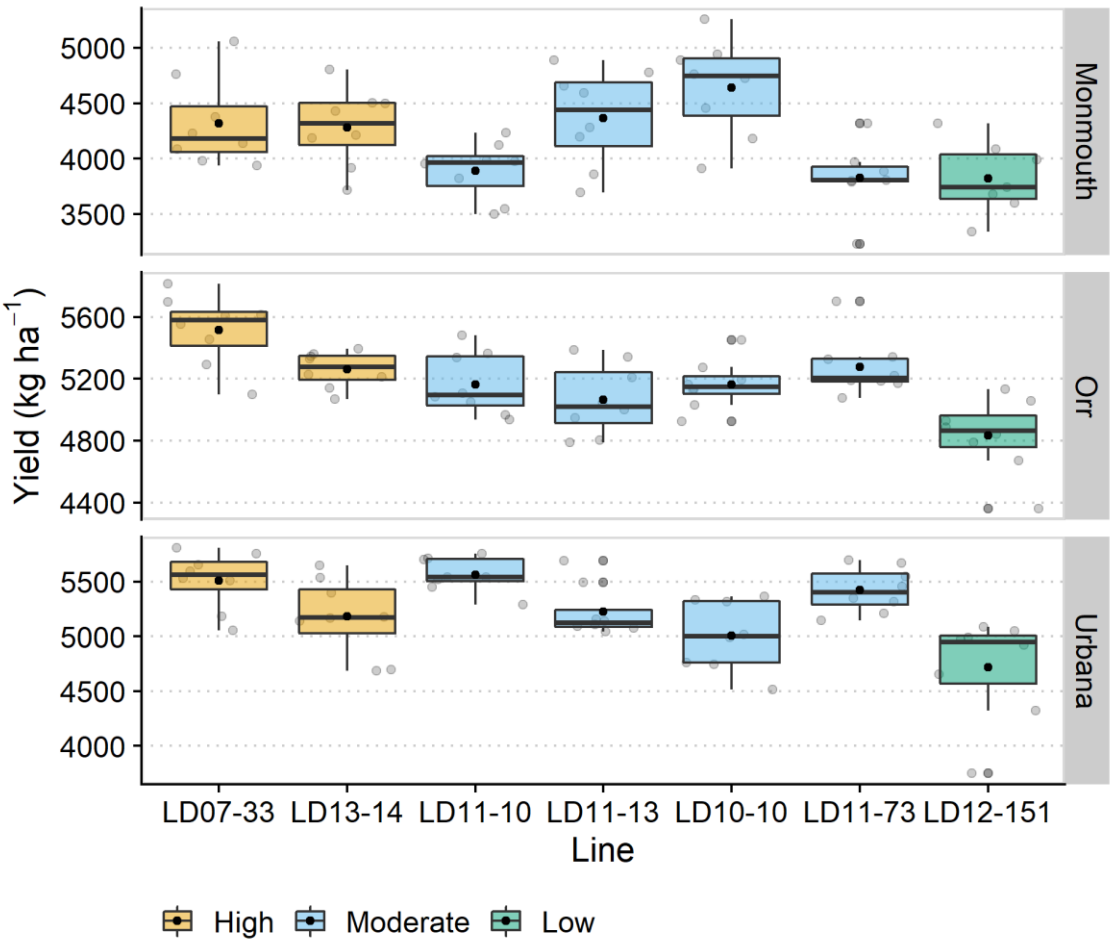

Figure 2. Yield distributions for soybean lines with different levels of partial resistance (PR) in multilocation field experiments in Illinois 2017. The center lines show the medians and black solid dots the means. Box limits indicate the 25th and 75th percentiles and whiskers extend 1.5 times the interquartile range. Solid gray dots represent outliers. Yield contrasts by PR group indicated a significant difference between the lines with high PR with the line with low PR in Monmouth (P = 0.046), Orr (P < 0.001), and Urbana (P < 0.001). Also, the lines with moderate PR had a significantly higher yield than the low PR line (P < 0.001) in Urbana, and the lines with high PR yielded significantly more than those with moderate PR (P = 0.003) in Orr. Abbreviations of lines are defined on Table 1.

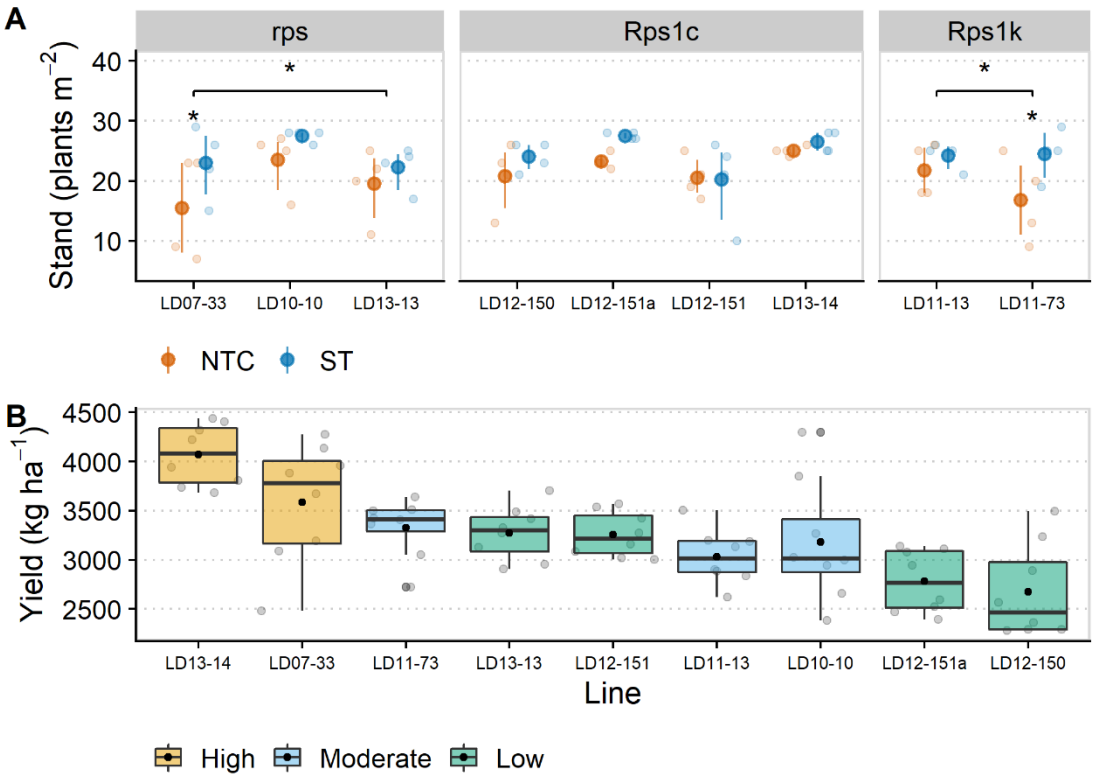

Figure 3. Effect of seed treatment and *Rps* gene on stand count at VC (A) and yield distributions (B) in a field trial in Urbana, IL, in 2018. A) Dots represent the mean stand of seed treatment plots (ST - blue) and non-treated control plots (NTC - orange). Vertical bars show confidence intervals of means. Panels with a bracket and asterisk represent a significant difference in the contrast between the non-treated control and the seed treatment when soybean lines were grouped by resistance gene. An asterisk above a soybean line represents a significant difference in the contrast between the non-treated control and the seed treatment for that soybean line only. B) The center lines show the medians and black solid dots the means. Box limits indicate the 25th and 75th percentiles and whiskers extend 1.5 times the interquartile range. Solid gray dots represent outliers. Yield contrasts by PR group indicated a significant difference between the lines with high PR and the lines with low PR ($P < 0.001$), and a significant difference between lines with high PR and those with medium PR ($P < 0.001$). Abbreviations of lines are defined on Table 1.

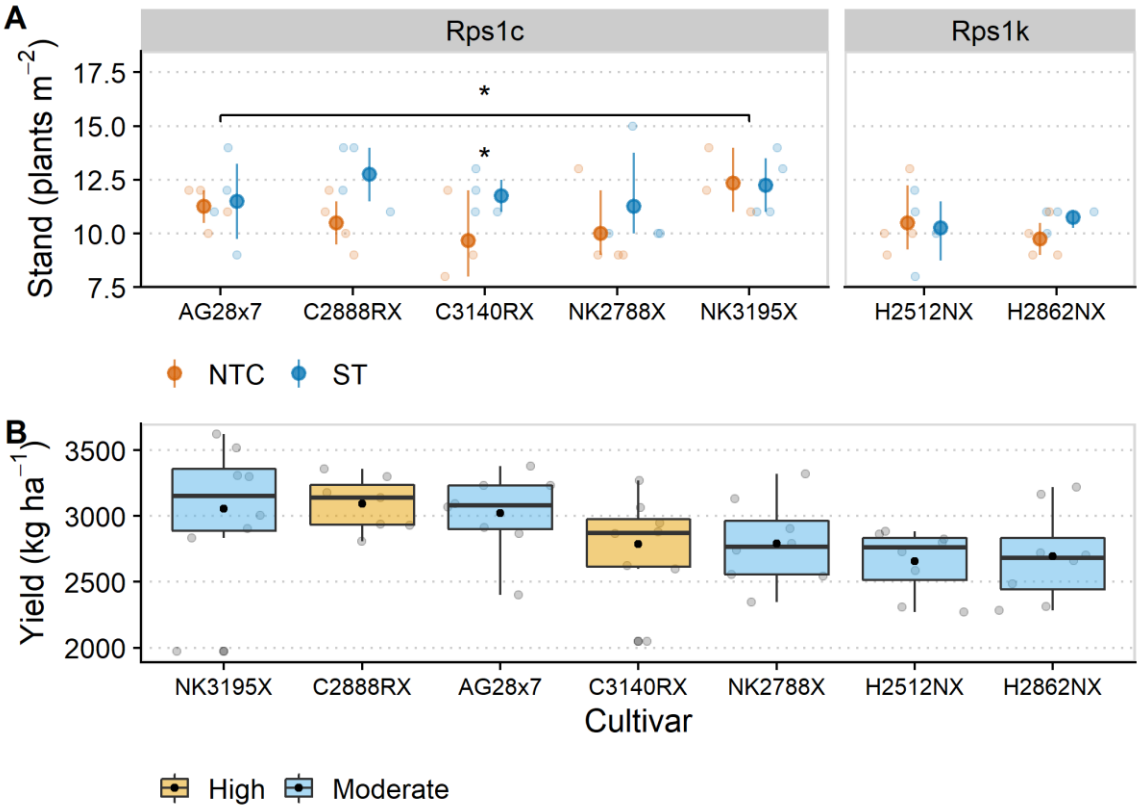

Figure 4. Effect of seed treatment and *Rps* gene on stand count at VC (A) and yield distributions (B) in a field trial in in Boone, IA, in 2018. A) Effect of seed treatment on cultivars with *Rps*1c or *Rps*1k on stand count. Dots represent the mean stand of seed treatment plots (ST - blue) or non-treated control plots (NTC - orange). Vertical bars show confidence intervals of means. Panels with a bracket and asterisk represent a significant difference in the contrast between the non-treated control and the seed treatment when cultivars were grouped by resistance gene. An asterisk above a cultivar represents a significant difference in the contrast between the non-treated control and the seed treatment for that cultivar only. B) Yield distributions for cultivar with different levels of partial resistance (PR). The center lines show the medians and black solid dots the means. Box limits indicate the 25th and 75th percentiles and whiskers extend 1.5 times the interquartile range. Solid gray dots represent outliers. There were not significant differences for yield between the cultivars with high and moderate PR.

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CHAPTER 2: CHARACTERIZATION OF PHYTOPHTHORA SPECIES ASSOCIATED WITH SOYBEAN IN ILLINOIS: COMPLEXITY, AGGRESSIVENESS AND FUNGICIDE SENSITIVITY

ABSTRACT

Phytophthora root and stem rot (PRR), caused by *Phytophthora sojae*, is one of the most devastating oomycete diseases of soybean in the Midwest U.S. Single resistant genes (*Rps*) are used to manage this pathogen, but *Ph. sojae* has adapted to *Rps* causing failure of resistance in many states. In addition to *Ph. sojae* recent reports indicate that *Phytophthora sansomeana* could also cause root rot on soybeans. Soil samples and symptomatic plants were collected across 40 Illinois counties between 2016 and 2018. *Ph. sojae* (77%) was more abundant than *Ph. sansomeana* (23%) across Illinois fields. Both species were characterized for virulence, aggressiveness and fungicide sensitivity. Virulence of all isolates was evaluated using the hypocotyl inoculation technique in 13 soybean differentials. Aggressiveness was evaluated in the greenhouse by inoculating a susceptible cultivar and measuring root and shoot dry weight. On average, *Ph. sojae* isolates were able to cause disease on six soybean differentials and was also the most aggressive species. *Ph. sansomeana* did not cause disease symptoms in both virulence and aggressiveness assays. No insensitive isolates to mefenoxam, metalaxyl, azoxystrobin or ethaboxam were detected. The characterization of the population of species associated to PRR will allow better management decisions regarding the use of resistance and fungicide seed treatments.

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is one of the most economically important crops in the state of Illinois (IL). In 2019, IL was the topmost soybean producer in the United States (US) contributing almost 15% (532 million bushels) of the total soybean production of the country (USDA-NASS 2020). Soybean production in IL and the US is severely affected by Phytophthora root and stem rot (PRR) caused by the oomycete *Phytophthora sojae* Kaufmann & Gerdemann (Allen et al 2017). PRR has been the fourth most destructive disease of soybean in the state for two decades (1996 to 2016), causing estimated losses of 945 million U.S. dollars during this period (Bandara et al. 2020). *Ph. sojae* is a soil-borne pathogen, which can infect soybean at any growth stage causing damping-off in seedlings and stem rot in older plants (Schmitthenner 2000). Planting cultivars with resistance to *Ph. sojae* (*Rps* genes) has been the primary method of management, but the pathogen has adapted to various deployed genes (*Rps*1a, *Rps*1c, *Rps*1k, *Rps*3a, and *Rps*6) and these genes are no longer effective in some regions of the US Midwest (Dorrance et al. 2016). Pathogen adaptation to *Rps* genes has been attributed to the selection pressure caused by the repeated planting of resistant cultivars and the evolution of avirulence (Avr) genes in the pathogen (Tyler 2007).

To monitor changes in virulence towards *Rps* genes and *Ph. sojae* pathotype diversity, surveys have been conducted across different states in the US Midwest (Dorrance et al. 2016; Dorrance et al. 2018). In these surveys, pathotypes of *Ph. sojae* were characterized based on their reaction with differentials carrying different *Rps* genes (Dorrance et al. 2008). Although PRR was first reported in Illinois in the 1950s, *Ph. sojae* pathotype (previously known as races) diversity was investigated for the first time only after more than four decades in 1997 (Kaufmann and Gerdemann 1958; Leitz et al. 2000). Seven pathotypes were identified from 33 isolates of which

3% were virulent to *Rps*1k and 17% to *Rps*1c, the most commonly deployed *Rps* genes (Leitz et al. 2000). A following survey of *Ph. sojae* pathotypes in IL in 2001 detected an increase in the pathotypes and proportion of isolates with virulence to the deployed resistance genes (Malvick and Grunden 2004). A total of 31 pathotypes of *Ph. sojae* were characterized from 121 isolates collected across IL out of which 60% were virulent to *Rps*1a, 42% to *Rps*1c, and 36% to *Rps*1k. In the most recent survey (2012-2013) conducted across 11 states including Illinois, 48 pathotypes were identified from 67 isolates in IL and 48% of the isolates were virulent to *Rps*1a, 42% to *Rps*1c, and 34% to *Rps*1k (Dorrance et al. 2016). Results from these surveys indicate that *Ph. sojae* pathotype diversity, virulence to deployed resistance genes used in commercial cultivars and overall isolate complexity (the number of *Rps* genes that an isolate is virulent to) has increased over time (Dorrance et al. 2016). Continued pathogen surveillance enables tracking of spatio-temporal diversity among *Ph. sojae* populations and detection of novel pathotypes, which in turn are important for recommendations regarding the deployment of specific genes in resistant cultivars in IL.

In addition to *Ph. sojae*, a second species, *Phytophthora sansomeana* E.M. Hansen & Reeser, has previously been reported as pathogenic to soybean (Hansen et al. 2009; Rojas et al. 2017a). Compared to the limited hosts of *Ph. sojae* , this species is also pathogenic to corn, peas, and Douglas fir (Hansen et al. 2009; L. X. Zelaya-Molina et al. 2009; Chang et al. 2017). Infection by *Ph. sansomeana* causes damping-off and root rot, but not stem rot, which is typical of *Ph. sojae* (Hansen et al. 2009; Rojas et al. 2017b). Although *Ph. sansomeana* was formally described in 2009, a second *Phytophthora* sp. causing PRR was first reported in Illinois during 2001-2002 from two counties and representing 4% of the total isolates recovered (Malvick and Gruden 2004). Pathotype characterization by inoculating *Phytophthora* sp. isolates on 11 soybean differentials

did not yield consistent results (Malvick and Gruden 2004). In a study to survey oomycete pathogens causing soybean seedling diseases, Rojas et al. (2017) isolated *Ph. sansomeana* from Illinois as well as from five other US states and Ontario, Canada. The survey detected *Ph. sansomeana* and multiple *Pythium* spp. but not *Ph. sojae*. Furthermore, *Ph. sansomeana* was among the most virulent oomycete species causing significant root rot when inoculated onto a susceptible soybean cultivar. Little is known about the prevalence of *Ph. sansomeana* in Illinois soils and its contribution to PRR, and if the current management practices for *Ph. sojae* would effectively manage *Ph. sansomeana*.

The most efficient way to manage PRR is host resistance with *Rps* genes, however, quantitative resistance and fungicide seed treatments along with cultural practices can also be valuable for integrated management when disease risk is high (Dorrance et al. 2009; Dorrance 2018; Cerritos-Garcia et al. 2021). In addition to *Rps* genes, soybean cultivars can have partial resistance, which is not race specific (Dorrance et al. 2003). Cultivars that include both qualitative and quantitative resistance are recommended in regions with high pathogen diversity within individual fields (Dorrance et al. 2003; Robertson 2009). Because quantitative resistance is expressed after emergence when the first unifoliate leaves appear, combining it with seed treatments is recommended for effective management (Dorrance and McCluren 2001). The phenylamides, mefenoxam and metalxyl, have been the primary fungicides used in seed treatments to control *Ph. sojae* (Dorrance 2018). Although there is a low risk of *Ph. sojae* developing resistance to these chemicals, it is important to monitor any changes in sensitivity, since they have been used for seed treatment for decades (FRAC 2020). Other compounds, such as ethaboxam and oxathiapiprolin, have recently been registered for use in soybean for oomycete control and are effective in controlling many oomycete species (Radmer et al. 2017). No baseline sensitivities for

these compounds for *Ph. sojae* or *Ph. sansomeana* have yet been established for the state of IL. Seed treatments also include QoI fungicides or strobilurins, which are broad-spectrum fungicides (Radmer et al 2017; Crop Protection Network 2020). There is a high risk of *Ph. sojae* developing resistance to these fungicides and they have even been reported ineffective against some *Pythium* species (Broders 2007; FRAC 2020). Determining the sensitivity of *Phytophthora* spp. to both targeted and broad-spectrum fungicides is important to make more accurate recommendations for the potential seed treatment combinations used in PRR management.

Surveys assessing pathotype diversity among *Ph. sojae* populations have been critical in evaluating the effectiveness of the most commonly deployed resistance genes and the emergence of more virulent and complex isolates of *Ph. sojae* in a soybean cultivation region. Therefore, the current study aims to characterize the population of *Phytophthora* spp. in Illinois soybean fields by evaluating virulence, aggressiveness, and fungicide sensitivity of the isolates recovered. Characterizing pathogens that contribute to the root and stem rot of soybeans will help make more accurate management recommendations regarding this disease.

MATERIALS AND METHODS

Sample collection

In fall 2016 and spring 2017, 50 soil samples were collected from 26 counties in IL (Figure 5). Fields either previously sampled in other surveys (Malvick and Grunden, 2004; Dorrance et al. 2016) within accessible locations were sampled. In each field, an area of about 7.62 x 53.4 m with favorable conditions for oomycetes (poorly drained, high humidity) was selected and 10 subsamples were collected following a zigzag pattern. Subsamples were collected at a depth of 15

cm and placed in a single collection bag per field. Soil samples were dried at room temperature and then stored in a cold room at 4°C until processing.

In the summer of 2018, 51 diseased soybean plants sampled from 19 counties were received from the Plant Clinic at the University of Illinois at Urbana-Champaign (UIUC) (Figure 5). All plants tested positive to the Phytophthora ImmunoStrip assay (Agdia, Elkhart, IN). Samples were stored in a cold room at 4°C and processed within two weeks.

Isolation and identification of isolates

Soil samples were ground using a Dynacrush soil mill (DC-5, Custom Laboratory Equipment Inc. Orange City, FL) and then subdivided into three to 10 plastic pots (15 cm diameter) (Dorrance et al. 2008). Soil in plastic pots were saturated with deionized, non-chlorinated water in the greenhouse, left overnight at 24-27°C, and drained the next day. After 24 to 48 hours, pots were placed in plastic bags and incubated in the dark at room temperature (~25°C). After incubation, 15 to 20 surface sterilized soybean seeds (immersion in 0.05% NaClO for 30 sec and then rinsed in sterile water) of the susceptible cultivar Sloan (no *Rps* gene and low partial resistance) was planted in the soil. Three days after planting, when seeds germinated, the pots were again saturated with water. Two weeks later, symptomatic seedlings that presented brown to tan lesions were selected for isolation.

For isolation of *Phytophthora* spp., symptomatic seedlings were washed with sterilized, distilled water (Dorrance et al. 2008), then, symptomatic tissue was cut into 1 to 2 cm sections, surface sterilized for 10 seconds in 0.5% NaClO and washed again with sterilized water. The symptomatic tissue was dried on a sterilized paper towel and plated on PBNIC selective media (Dorrance et al. 2008). Plates were incubated at room temperature and monitored daily for mycelial growth. Plates with mycelia that resembled oomycete species were transferred to either lima bean

agar (LBA) or V8 juice agar (Dorrance et al. 2008). Isolates that resembled oomycetes species based on morphology were identified and stored in vials with V8 juice agar at 15°C for further identification. Oomycetes were isolated from symptomatic plants received from the UIUC Plant Clinic in 2018 using the same procedure with the exception that plants were first washed with tap water and soap to remove soil particles.

The identity of the isolates was verified by amplification and sequencing of internal transcribed spacer (ITS) region. For this, 3 mm plugs from cultures in the storage vials were transferred to an Erlenmeyer flask containing 125 to 150 ml of V8 broth and isolates were cultured for one week on an orbital shaker (New Brunswick Scientific, Edison, NJ). After a week, approximately 100 milligrams of mycelia were transferred to FastDNA Lysing Matrix A tubes and DNA was extracted using the FastDNA SPIN Kit protocol (MP Biomedicals, Solon, OH). Amplification of the ITS region and sequencing was done using ITS 4 (5′-TCCTCCGCTTATTGATATGC) and ITS 5 (5′-GGAAGTAAAAGTCGTAACAAGG) primers (White et al. 1990). The PCR product was cleaned using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and samples were submitted for Sanger sequencing to the UIUC Core sequencing facility. All isolates recovered from soil samples were sequenced. For isolates from plant samples, only those identified as *Phytophthora* spp. based on morphology and PCR, were sequenced. The sequenced data were initially subjected to a BLAST alignment on the Phytophthora-ID website database (http://phytophthora-id.org/)(Grünwald et al. 2011) and isolates that remained unidentified were further blasted against the NCBI nr database for the closest match.

Pathotype evaluation

Pathotypes for each isolate were determined by inoculating a set of 13 differentials using the hypocotyl inoculation technique (Dorrance et al. 2008). The following differentials were used: Williams (universal susceptible, *Rps*), Harlon (*Rps*1a), Harosoy13XX (*Rps*1b), Williams79 (*Rps*1c), Williams82 (*Rps*1k), L76-1988 (*Rps*2), L83-570 (*Rps*3a), PRX-146-36 (*Rps*3b), PRX-145-48 (*Rps*3c), L85-2352 (*Rps*4), L85-3059 (*Rps*5), Harosoy62XX (*Rps*6), and Harosoy (*Rps*7) (Dorrance et al. 2004). Twenty seeds of each differential were surface sterilized with 1% bleach for 30 seconds and planted in 1020-tray inserts filled with coarse vermiculite. After planting, to avoid fungal contamination, the trays were sprayed with a 0.6% benomyl solution (0.6 g Benlate 50 WP) and placed in the greenhouse at 25-28°C under a 16-hour photoperiod. Trays were watered and sprayed daily with the benomyl solution. One week after planting, 10 to 15 seedlings of each differential were selected for inoculation.

Inoculum was prepared as described by Dorrance et al 2008. Briefly, *Phytophthora* spp. isolates were sub-cultured in LBA or V8 media and incubated at room temperature. After one week, each culture was transferred to a 10 ml syringe and forced through the syringe into another syringe with an 18-gauge needle, which was used for inoculation. One week old soybean seedlings were inoculated by making a 1 cm slit in the hypocotyl with the 18-gauge needle and injecting 0.2 to 0.4 ml of culture slurry on and around the slit. The slit was the covered with Parafilm to maintain humidity. Inoculated seedlings were incubated in a moisture chamber (95% humidity) for 48 hours at 20-22 °C in the dark, transferred to the greenhouse (25-28°C, 16-hour photoperiod) and evaluated for disease after seven days. Reactions were scored as resistant when ≤ 25% of the seedlings were dead or as susceptible when ≥ 75% of the seedlings were dead.

Aggressiveness evaluation

We used a modified version of the layer test to evaluate the aggressiveness of *Phytophthora* spp. isolates (Stewart and Robertson 2011; Rojas et al. 2017). Eleven-centimeter wide Jiffypots (Jiffy, Shippagan, Canada) with three holes at the bottom for drainage were used for the experiment. The pots were filled with 100 ml of coarse vermiculite, followed by the inoculum and a second layer of 200 ml of vermiculite. Eight seeds of the susceptible cultivar, Sloan, were placed about 2.5 cm over the inoculum and then covered with a final layer of 200 ml of vermiculite. Inoculum consisted of a *Phytophthora* spp. culture grown in V8 media for two weeks at room temperature. The experiment was arranged in a completely randomized design with three replicates per treatment (isolates and controls) and pot as the experimental unit. Treatments including a non-inoculated V8 agar layer (control agar) and only vermiculite (control none) served as controls. The experiment was repeated three times.

After planting, the pots were saturated with water until runoff and placed in the greenhouse (~24°C, 16-hour photoperiod). The pots were watered daily. Two weeks after germination, seedlings were removed from the pots, and the roots were washed with tap water to remove the vermiculite. Shoots and roots were separated, placed in paper bags and dried at 50°C in a laboratory oven (Precision Thelco oven, Thermo Fisher Scientific). The dry weight of shoots and roots was measured 48 h after drying. Germination varied by pot and therefore, averages by the number of seedlings per pot were used for analysis. Average root and shoot dry weights were estimated by dividing the total root dry weight and shoot dry weight by the number of seedlings per pot, respectively.

Fungicide sensitivity

To determine the sensitivity of *Phytophthora* spp. isolates to fungicides, we selected one *Ph. sojae* isolate from each county (n = 9) and all *Ph. sansomeana* isolates (n = 7). We used poison plate assays to evaluate fungicide sensitivity of isolates. Isolates were tested for sensitivity to azoxystrobin (Syngenta Crop Protection, Greensboro, NC), ethaboxam (Valent U.S.A. LLC, Walnut Creek, CA), mefenoxam (Syngenta Crop Protection, Greensboro, NC), and metalaxyl (Gustafson, Plano, TX). Technical grade fungicides were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions of 10 mg of a.i./ml. Then, we diluted stock solutions and added them to LBA medium (50-55˚C) to obtain final concentrations of 0, 0.1, 0.5, 0.1, 1 and 10 μg/ml for azoxystrobin; 0, 0.001, 0.01, 0.05, 0.1 and 1 μg/ml for ethaboxam; and 0, 0.01, 0.05, 0.1, 0.5 and 1 μg/ml for mefenoxam and metalaxyl, respectively. One ml of DMSO was added to the medium as control (dose 0). For azoxystrobin, 25 mg of salicylhydroxamic acid (SHAM) (Fisher Scientific) was added to the medium to avoid the alternative oxidase pathway (Broders et al. 2007). The amended medium was poured into petri dishes (60 × 15 mm). Fungicide dilutions and SHAM concentrations were determined based on preliminary experiments (data not shown).

Phytophthora sojae (n = 9) and *Ph. sansomeana* (n = 7) were grown in V8 juice medium at room temperature. After two weeks of growth, agar plugs (4mm diameter) of each isolate were transferred to the center of the fungicide amended plates. Plates were incubated at 25˚C with a 10 h photoperiod (Precision Incubator Model 815, Thelco). Colony growth was determined after seven days by measuring colony diameter twice at right angles. Percent growth relative to the control was calculated by dividing the length of colony of the fungicide amended plate by the average length of the control plates. Each isolate was replicated three times per concentration, and the experiment was repeated twice.

Data analysis

The pathotype of each isolate, pathotype frequency and complexity (number of *Rps* genes that an isolate can overcome and cause disease) were determined using the package 'hagis' (McCoy et al. 2019) as implemented in R version 4.0.2. A linear mixed model was fitted to the aggressiveness data (root dry weight and shoot dry weight) with species and isolates as fixed effects and experiment as random effect. Models were fit using package 'lme4' and then the ANOVAs were conducted with the package 'lmerTest' (Bates et al. 2015; Kuznetsova et al. 2017). Residual plots were generated with the package 'ggResidpanel' to check model assumptions (Goode and Rey 2019). Estimated marginal means were obtained and mean separation was done using Tukey's HSD with the package 'emmeans' (Lenth 2020). For fungicide sensitivity data, the effective fungicide concentration to reduce the growth by 50% (EC_{50}) was calculated for each isolate using the log-logistic four-parameter model in the package 'drc' (Ritz et al. 2015). Pearson correlation was performed to determine if there was any relationship between complexity and aggressiveness. All the data and code used for analysis are available at https://github.com/danielcerritos/phytophthora.

RESULTS

Identification of oomycetes from symptomatic soybean plants and soybean field soils in IL

A total of 148 oomycetes were recovered from symptomatic soybean plants and field soils collected from 32 counties. The recovered isolates were categorized into 12 species based on morphology and ITS sequences. Overall, *Pythium* spp. dominated both field soils and diseased plants. *Pythium ultimum* var. *ultimum* (42%) was the most abundant species, followed by *Ph. sojae* (24%) and *Ph. sansomeana* (7%). The remaining nine species included: *Pythium yorkensis*,

Pythium pleroticum, *Pythium aphanidermatum*, *Pythium torulosum*, *Pythium vexans*, *Pythium ultimum* var. *sporangiiferum*, *Pythium acanthophoron*, *Pythium irregulare*, and *Pythium acrogynum*. A total of 31 isolates were identified as *Phytophthora* spp., which were recovered from eight of the 50 soil samples (16%) and from eight of the 51 plant samples (16%) in 12 counties out of the 40 sampled (Table 2). *Ph. sojae* and *Ph. sansomeana* were recovered from both soil and symptomatic plants. *Ph. sojae* was recovered from nine fields in 10 counties with more than one isolate recovered from seven fields. *Ph. sansomeana* was recovered from five fields in five counties and more than one isolate were recovered from two of these fields.

Some soil samples contained both *Phytophthora* and Pythium spp. While *Ph. sojae* was isolated with *P. ultimum* var. *ultimum* and *P. acanthophoron* from one of the soil samples, it co-occurred with *P. ultimum* var. *ultimum* and *P. torulosum* in another soil sample. *Ph. sojae* and *Pythium* spp. were not recovered from samples containing *Ph. sansomeana*.

Virulence assays

Pathotype(s) for 24 *Ph. sojae* isolates were characterized by infecting 12 soybean differentials with known *Rps* genes and one susceptible control. Sixteen pathotypes were identified from (Table 2). Only 8% of the isolates were virulent to *Rps*6 compared to 21% virulent to *Rps*4 and 25% to *Rps*3b (Figure 6A). All the isolates were virulent to *Rps*1b and *Rps*5. More than 50% of the isolates were virulent to *Rps*1k, the most commonly deployed gene in IL in addition to *Rps*7 and *Rps*3c. Thirty three percent of the *Ph. sojae* isolates were virulent to *Rps*1a and *Rps*1c. The complexity of isolates (number of differentials an isolate can cause disease on) ranged from four to nine with mean complexity of six. No isolate was virulent to all differential lines tested (Figure 6B).

Of the 16 pathotypes of *Ph. sojae*, five were identified more than once, and represented 80% of the total isolates (Table 2). Of these five pathotypes, only a single pathotype (1b, 1k, 7, 5) was recovered in more than one field. Within each field, pathotypes recovered ranged from one to four. Out of the fields from which more than one *Ph. sojae* isolate was recovered, 43% contained isolates with a single pathotype, 43% with two pathotypes and 14% with four pathotypes. In the fields where we recovered two pathotypes of *Ph. sojae*, isolate complexity ranged from four to eight, whereas for the field with four pathotypes, complexity ranged from four to nine.

No pathotypes were identified for the *Ph. sansomeana* isolates using the *Ph. sojae* differentials. None of the *Ph. sansomeana* isolates were able to cause disease on the *Ph. sojae* susceptible control differential (Williams). Interestingly, 43% of the *Ph. sansomeana* isolates were virulent to L83-570 (*Rps*3a) and 14% to Williams79 (*Rps*1c) while 14% percent was virulent on both differentials.

Aggressiveness of *Phytophthora* spp. on soybean

Aggressiveness of *Phytophthora* spp. isolates was assessed by infecting a susceptible cultivar and measuring root dry weight and shoot dry weight two weeks after germination. The average temperature of the three experiments was 24.3°C (min: 20.89 - max: 29.6°C). Overall, *Ph. sojae* was more aggressive than *Ph. sansomeana* (Figure 7). Significant differences between species were observed for both root and shoot dry weight ($P < 0.001$). *Ph. sojae* was significantly different from both controls and *Ph. sansomeana* for both root and shoot weight. No difference was observed between the controls and *Ph. sansomeana* for root or shoot weight. On average, *Ph. sojae* reduced root weight by 54% and shoot weight by 48% compared to the control. This contrasts with *Ph. sansomeana*, which reduced root and shoot weight by 10% and 15%, respectively.

Differences among isolates were observed for both root weight ($P < 0.001$) and shoot weight ($P = 0.0294$). Isolate aggressiveness was compared against the control agar. In addition, we observed a difference between the two controls for shoot weight. For root weight, 23 *Ph. sojae* isolates (96%) were significantly different from the control and only two were significantly different from the most aggressive isolate (Figure 8). The most aggressive isolate reduced root weight by 73% compared to the least aggressive, which reduced weight by 27%. Isolates not significantly different from the most aggressive isolate reduced root weight between 40 and 72%. The two isolates significantly different from the most aggressive isolate but not the control and only reduced root weight on average by 37%.

For shoot weight, 22 *Ph. sojae* isolates (92%) were significantly different from the control but did not differ significantly from the most aggressive isolate (Figure 8). The most aggressive isolate reduced shoot weight by 59% compared to a 15% reduction by the least aggressive isolate. Isolates that did not differ significantly from the most aggressive isolate reduced shoot weight between 41% and 58%. The isolate for which significant differences were not observed when compared with either the most aggressive isolate or the control reduced shoot weight by 39% on average.

None of the *Ph. sansomeana* isolates were significantly different from the control agar for both root and shoot weight (Figure 8). Among the *Ph. sansomeana* isolates, all isolates differed significantly from the most aggressive isolate for both root and shoot weight. *Ph. sansomeana* isolates reduced root weight between 2% and 15% and shoot weight between 7% and 21%.

Correlation between isolate complexity and aggressiveness of *Ph. sojae*

Pearson correlation analysis was performed to determine if pathotype complexity of *Ph. sojae* isolates correlated with aggressiveness. A weak positive significant correlation was observed

between isolate complexity with root weight (r = 0.18, *P* = 0.009) and shoot weight (r = 0.16, P = 0.015). Isolates with a higher complexity tended to be the less aggressive (Figure 9). A moderate and significant correlation was detected between root and shoot weight (r = 0.54, *P* < 0.001).

Sensitivity of *Phytophthora* isolates to fungicides

All isolates of *Ph. sojae* (*n* = 9) and *Ph. sansomeana* (*n* = 7) were sensitive to the four fungicides tested (Figure 10) as indicated by reduction in growth. On average, the highest EC_{50} values were observed for azoxystrobin (1.5 µg/ml), followed by mefenoxam (0.06 µg/ml), metalaxyl (0.05 µg/ml) and ethaboxam (0.02 µg/ml) indicating that ethaboxam caused the greatest reductions in growth of *Phytophthora* isolates. Sensitivity to ethaboxam varied by species (Figure 6). On average, higher EC_{50} values were observed for *Ph. sansomeana* (0.023 µg/ml) compared *Ph. sojae* (0.008 µg/ml). *Ph. sojae* was also more sensitive to azoxystrobin (1.2 µg/ml) compared to *Ph. sansomeana* (1.9 µg/ml). The response to mefenoxam and metalaxyl was similar for both species. Average EC_{50} values for mefenoxam were 0.060 µg/ml for *Ph. sojae* and 0.061 µg/ml for *Ph. sansomeana*. For metalaxyl, average EC_{50} values were 0.056 µg/ml for *Ph. sojae* and 0.051 µg/ml for *Ph. sansomeana*. Although no insensitive isolates were detected, the distribution of EC_{50} values show that some isolates were less sensitive to all the fungicides tested (Figure 10). A *Ph. sansomeana* isolate (18PR005) showed the highest resistance against all the fungicides and had the highest EC_{50} values (Figure 10).

DISCUSSION

Understanding the dynamics of pathogen diversity and virulence in a region is critical towards informed disease management. The current study sought to identify and characterize populations of *Phytophthora* spp. from soybean plants with PRR and field soils from IL in an

attempt towards continued surveillance. Interestingly, soil and plant samples collected from across 40 counties were dominated by *Pythium* spp. (79%), whereas *Phytophthora* spp. isolates consisted of 21% of the total isolates recovered. *Pythium ultimum* var. *ultimum* (42%) was identified as the most frequent species followed by *Ph. sojae* (24%) and *Ph. sansomeana* (7%). Pathotype characterization of *Ph. sojae* resulted in identification of 16 pathotypes and on average, isolates were able to cause disease on six *Rps* genes. There was an increase in pathogen complexity compared to previous surveys. *Ph. sansomeana* caused little seedling damage under conditions were damage from *Ph. sojae* was observed. Both species were sensitive to mefenoxam, metalaxyl, azoxystrobin and ethaboxam.

Isolates collected in this study were more complex compared to the past surveys suggesting an increase in virulence to several resistance genes in IL. Compared to the most recent survey in IL, isolate mean complexity increased from 4.4 to 6.0 (Dorrance et al. 2016). Additionally, pathotypes, such as race 0 (virulent only to universal susceptible) and race 1 (Rps7), that were common in the past surveys, were not detected in the current study (Hartman et al. 1997; Malvick and Gruden 2004; Dorrance et al. 2016). While the complexity of *Ph. sojae* in IL has increased, an increase in virulence to commonly deployed *Rps* genes (*Rps*1a, *Rps*1c and *Rps*1k) was not observed for all genes. In the most recent survey (2012-2013), an increase in virulence to all of the deployed genes was reported compared to the survey in 2001-2002 done in Illinois (Malvick and Gruden 2004; Dorrance et al. 2016). Compared to the last survey (Dorrance 2016) we observed an increase in virulence to *Rps*1b, *Rps*1k and *Rps*3a, but a decrease for *Rps*1a, *Rps*1c and *Rps*6. Although only 33% of our isolates were virulent to *Rps*1a and *Rps*1c, isolates with virulence to these genes were found in five of the nine fields (55%) from where *Ph. sojae* was recovered. Virulence to *Rps*1k was detected in eight of the nine fields (89%). These three *Rps* genes (*Rps*1a,

*Rps*1c and *Rps*1k) are the most commonly deployed resistance genes in soybean cultivars in IL (Slaminko et al. 2010).

Reintroduction and rotation of the less commonly deployed genes, *Rps*3a and *Rps*6, have been proposed for long-term management of PRR (Dorrance et al. 2016; Yan and Nelson 2019). Dorrance et. al. (2016) reported that less than 10% of the isolates recovered in five of the 11 states surveyed for *Ph. sojae* pathotype diversity were virulent to these genes. However, in Illinois, more than 40% of isolates could overcome resistance from *Rps*3a. In the current study, *Rps*6 was the most effective gene with only two isolates (8%) virulent to it. Because these two isolates were recovered from the same field, *Rps*6 may still be effective in majority of the fields in IL. Although the past survey by Dorrance et al. (2016) reported a higher number of isolates that could overcome *Rps*6, it was still the most effective gene among the deployed genes. Previous surveys in the state also reported only a few isolates that could overcome this gene (Hartman et al. 1997; Malvick and Gruden 2004). Cultivars with *Rps*6 could be a possibility to manage PRR, however, cultivars with *Rps*6 are uncommon in IL (Slaminko et al. 2010). Between 2003 and 2008, only two out of more than 3,000 cultivars that enter the VIPS econtained *Rps*6 (Slaminko et al. 2010). Between 2009 and 2020, more than 2,000 cultivars entered the program and none of these had *Rps*6 (University of Illinois, VIPS). Gene stacking of *Rps*3a and *Rps*6 with *Rps*1c or *Rps*1k has also been proposed (Dorrance et al. 2016). Gene stacking is also uncommon in IL, but some companies have reported cultivars with *Rps*1c and *Rps*3a (University of Illinois, VIPS). Although more than 45% of our isolates could overcome *Rps*3a, only 13% isolates recovered from two fields were virulent to both *Rps*1c and *Rps*3a. Deployment of two genes (*Rps*1c and *Rps*3a) in soybean cultivars maybe essential for long-term management of PRR in IL, but cultivar selection may come with the cost of reincorporating these genes in breeding programs.

Ph. sojae surveys are mostly focused on isolate virulence and aggressiveness of isolates is normally not assessed. The current study evaluated aggressiveness of *Ph. sojae* isolates and found a weak positive correlation between aggressiveness and isolate complexity such that the least aggressive isolates were more complex (7-9). Despite the positive correlation between complexity and aggressiveness, not all isolates with higher complexity were the less aggressive. We did not find any evidence that having more virulence factors could negatively influence fitness, at least for aggressiveness. If there was a cost for carrying additional virulence genes, frequency of these genes should decrease over time in the pathogen population (Zhan and McDonald 2013). Complexity has instead increased over time and virulence to specific genes is maintained in populations across US Midwest (Dorrance et al. 2016). Although most *Ph. sojae* isolates were not significantly different from the most aggressive isolate, differences among isolate aggressiveness were detected, which may have implications in PRR management using partial resistance. *Ph. sojae* isolates used in screening for partial resistance should be evaluated for their aggressiveness.

Ph. sojae is the primary causal agent of PRR, but *Ph. sansomeana* has recently been reported in soybean producing states (Rojas et al. 2017; Tande et al. 2020). In the current study, *Ph. sansomeana* was isolated from symptomatic soybean plants and field soils. However, it occurred at lower frequencies compared to *Ph. sojae* and failed to cause PRR symptoms in both pathotype and aggressiveness assays. Rojas et al. (2017) reported this species as one of the most aggressive oomycetes species in seed and seedling assays at 20°C, but disease did not manifest at 15°C. In contrast, Hansen et al. (2009) reported disease symptoms at both of these temperatures, but higher disease severity at 15°C. In this study, average temperatures of both experiments were close to 25°C with maximum temperatures up to 30°C. It has been reported that aggressiveness of

oomycetes can vary depending on the temperature (Matthienssen et al. 2016; Rojas et al. 2017). This could possibly explain absence of disease symptoms in the current study.

While the current study detected both *Ph. sojae* and *Ph. sansomeana* associated with PRR in Illinois, recovery from both soil and plant samples was low compared to the surveys conducted previously. *Phytophthora* spp. were isolated from 16% of soil samples in the current study. In contrast, Dorrance et al. (2016) isolated *Ph. sojae* (n = 67) from 67% soil samples collected from across 11 states in the US and Malvick and Grunden (2004) isolated *Ph. sojae* from 42% field soils in IL. Lower isolate collection was expected in the current survey because our sampling was less intensive. Soil samples were collected in 50 fields from 26 counties compared to 76 fields from 56 counties in the survey by Dorrance et al. (2016) and 80 fields from 32 counties in Malvick and Grunden (2004) study, respectively. Additionally, both studies baited soils twice, unlike the current study in which soils were baited only once. A second round of soil baiting might have resulted in a higher percentage of *Phytophthora* spp. It is however noteworthy that both field soils and symptomatic plants were dominated by *Pythium* spp. in the current survey in IL.

Although the overall recovery of *Phytophthora* spp. was low, at least one isolate was recovered from 67% of the samples. Most of these isolates were identified as *Pythium* spp. This is not surprising because more than 40 *Pythium* species are pathogenic to soybeans (Rojas et al. 2017a). *Ph. sojae* and *Pythium* spp. were isolated from the same samples and it becomes important to characterize these isolates to evaluate their pathogenicity to soybeans. Seedling diseases of soybeans are caused by a complex of *Pythium* species symptoms of which are very similar to those caused by *Ph. sojae* early in the season (Broder et al. 2007).

TABLE AND FIGURES

Table 2. *Phytophthora* spp. isolates recovered from Illinois

Isolate ID	Species	County	Year	Pathotype
16PR018B.1	*Phytophthora sansomeana*	Kankakee	2016	
16PR024C.1	*Phytophthora sansomeana*	Grundy	2016	
16PR024C.2	*Phytophthora sansomeana*	Grundy	2016	
17PR006L.1	*Phytophthora sansomeana*	Macon	2017	
18PR003	*Phytophthora sansomeana*	Champaign	2018	
18PR004	*Phytophthora sansomeana*	Champaign	2018	
18PR005	*Phytophthora sansomeana*	Champaign	2018	
16PR009.1	*Phytophthora sojae*	Champaign	2016	1b, 7, 2, 3b, 3c, 5
16PR009.2	*Phytophthora sojae*	Champaign	2016	1b, 7, 2, 3a, 3b, 3c, 5
16PR027C.1	*Phytophthora sojae*	Livingston	2016	1a, 1b, 1c, 1k, 3c, 4, 5
16PR027C.2	*Phytophthora sojae*	Livingston	2016	1a, 1b, 1c, 1k, 3c, 4, 5
17PR009A.1	*Phytophthora sojae*	Franklin	2017	1b, 6, 7, 3c, 4, 5
17PR009A.2	*Phytophthora sojae*	Franklin	2017	1b, 6, 7, 3c, 4, 5
17PR013G.3	*Phytophthora sojae*	Crawford	2017	1a, 1b, 1c, 1k, 7, 3a, 3b, 3c, 5
17PR018F.1	*Phytophthora sojae*	Boone	2017	1a, 1b, 1c, 1k, 7, 5
17PR018F.2	*Phytophthora sojae*	Boone	2017	1b, 1k, 7, 5
17PR018H.1	*Phytophthora sojae*	Boone	2017	1a, 1b, 1c, 1k, 7, 5
17PR018J.3	*Phytophthora sojae*	Boone	2017	1b, 1k, 7, 5
18PR001	*Phytophthora sojae*	Vermilion	2018	1a, 1b, 1c, 1k, 7, 2, 3b, 5
18PR007	*Phytophthora sojae*	Massac	2018	1a, 1b, 1c, 1k, 2, 3a, 3c, 5
18PR006	*Phytophthora sojae*	Massac	2018	1a, 1b, 1c, 1k, 7, 2, 3a, 5
18PR008	*Phytophthora sojae*	Knox	2018	1b, 7, 3a, 3c, 5
18PR010	*Phytophthora sojae*	Knox	2018	1b, 7, 3a, 3c, 5
18PR009	*Phytophthora sojae*	Knox	2018	1b, 7, 3a, 5
18PR011	*Phytophthora sojae*	Jefferson	2018	1b, 1k, 7, 5
18PR012	*Phytophthora sojae*	Jefferson	2018	1b, 1k, 7, 5
18PR016	*Phytophthora sojae*	Knox	2018	1b, 7, 3a, 3c, 5
18PR014	*Phytophthora sojae*	Jefferson	2018	1b, 1k, 3c, 5
18PR015	*Phytophthora sojae*	Jefferson	2018	1b, 1k, 7, 2, 3a, 3b, 3c, 5
18PR013	*Phytophthora sojae*	Knox	2018	1b, 3a, 3c, 5
18PR017	*Phytophthora sojae*	Jefferson	2018	1b, 1k, 7, 2, 3a, 3b, 3c, 4, 5

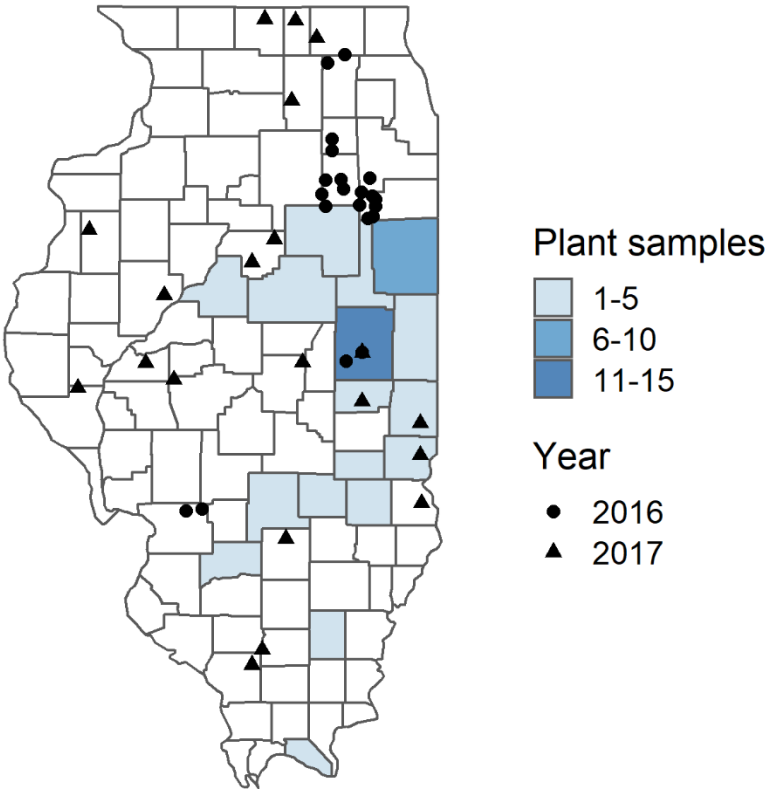

Figure 5. Map of fields where soil samples were collected in 2016 and 2017 and counties from where symptomatic plants were received in 2018.

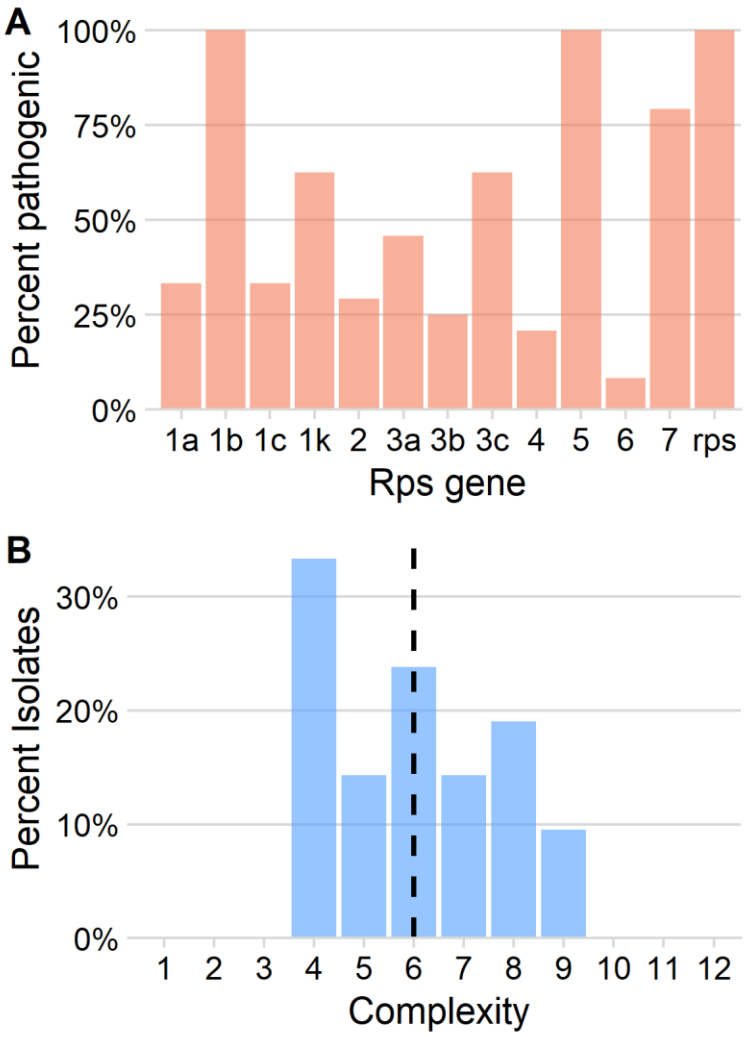

Figure 6. (A) Percentage of *Phytophthora sojae* isolates (n = 24) virulent to differentials with different *Rps* genes. **(B)** Frequency distribution of complexity (the number of *Rps* genes that an isolate is virulent to) of *Ph. sojae* isolates.

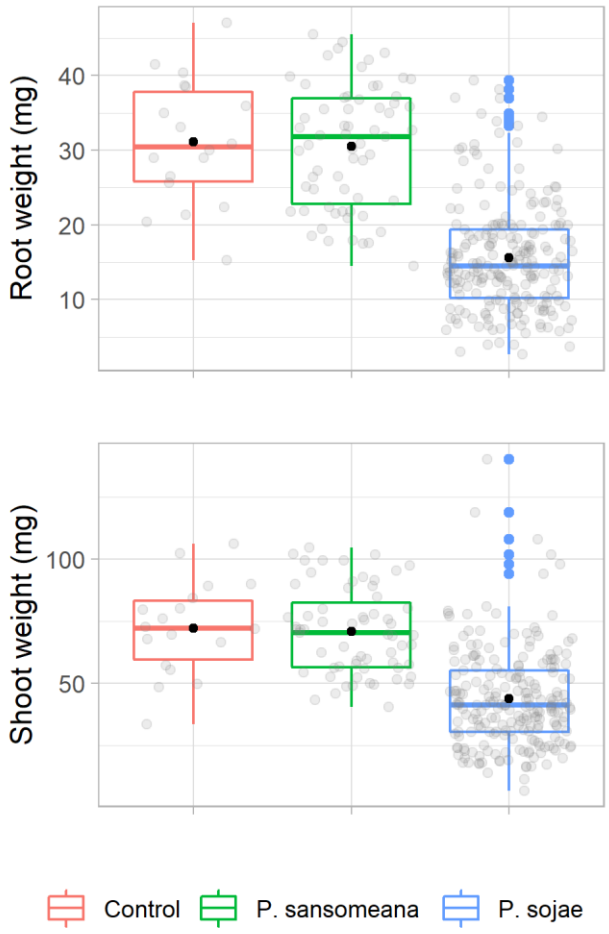

Figure 7. Distribution of root and shoot dry weight of the susceptible cultivar, Sloan (no Rps), inoculated with isolates of *Phytophthora sansomeana* (n = 7) and *Phytophthora sojae* (n = 24). Center lines represent the medians and black solid dots the means. Box limits indicate the 25th and 75th percentiles and whiskers extend 1.5 times the interquartile range. Solid colored dots represent outliers.

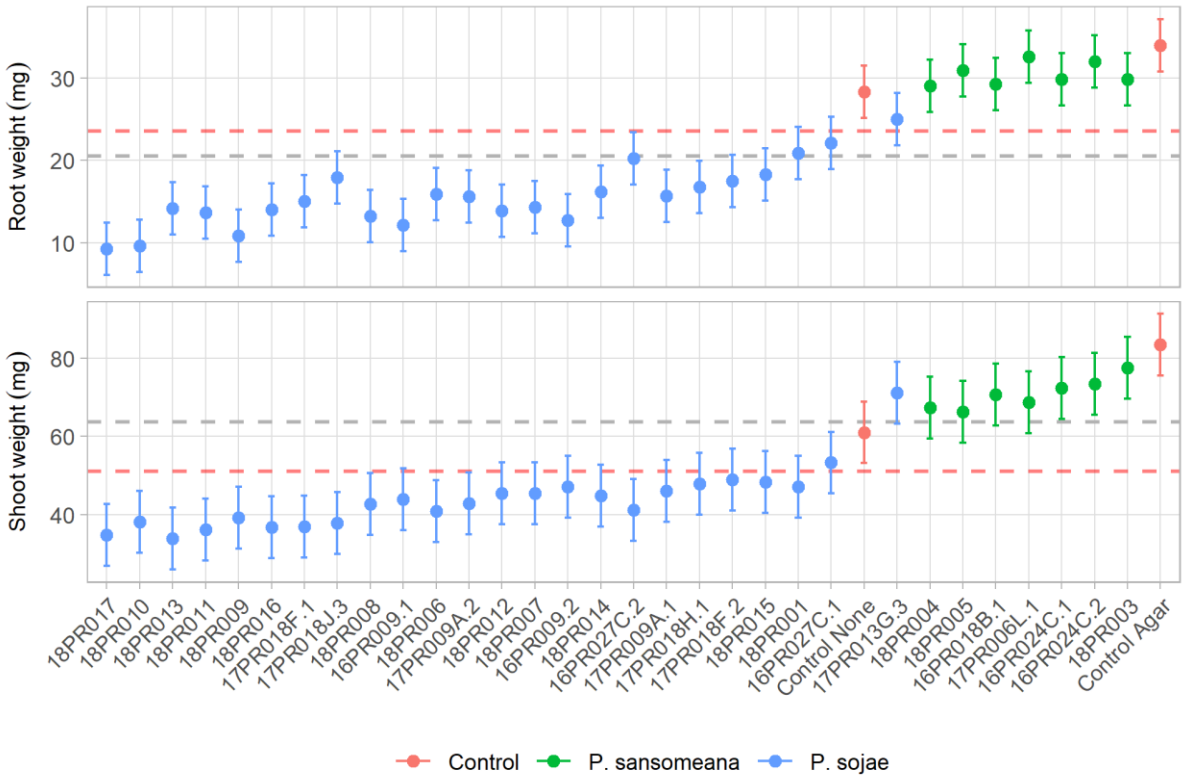

Figure 8. Mean root and shoot dry weight of the susceptible cultivar, Sloan (no Rps), inoculated with isolates of *Phytophthora sansomeana* and *Phytophthora sojae*. Verticals lines represent the mean standard error. Means under the gray dotted line are significantly different from the control agar and means over the red dotted line are significantly different from the most aggressive isolate (Tukey's HSD, $P < 0.05$).

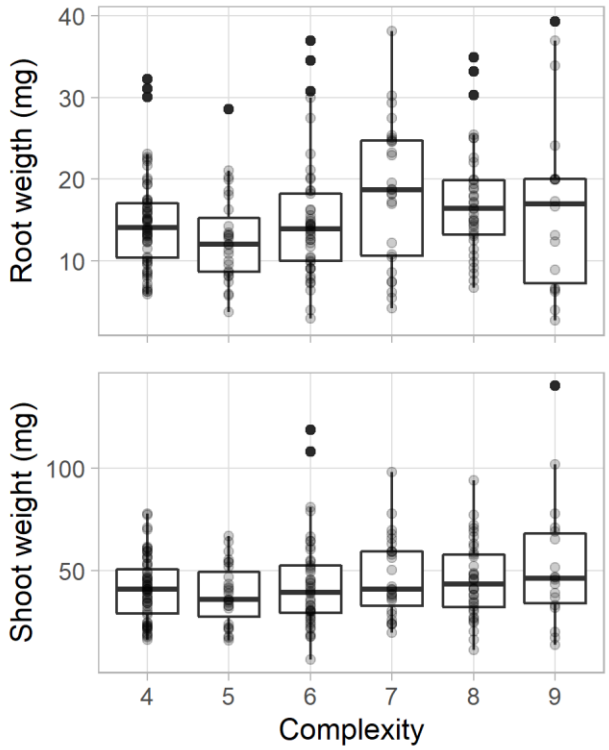

Figure 9. Distribution of root and shoot dry weight of the susceptible cultivar, Sloan (no Rps), inoculated with isolates of *Phytophthora sojae* (n = 24) grouped by complexity. Center lines represent the medians and black solid dots the means. Box limits indicate the 25th and 75th percentiles and whiskers extend 1.5 times the interquartile range.

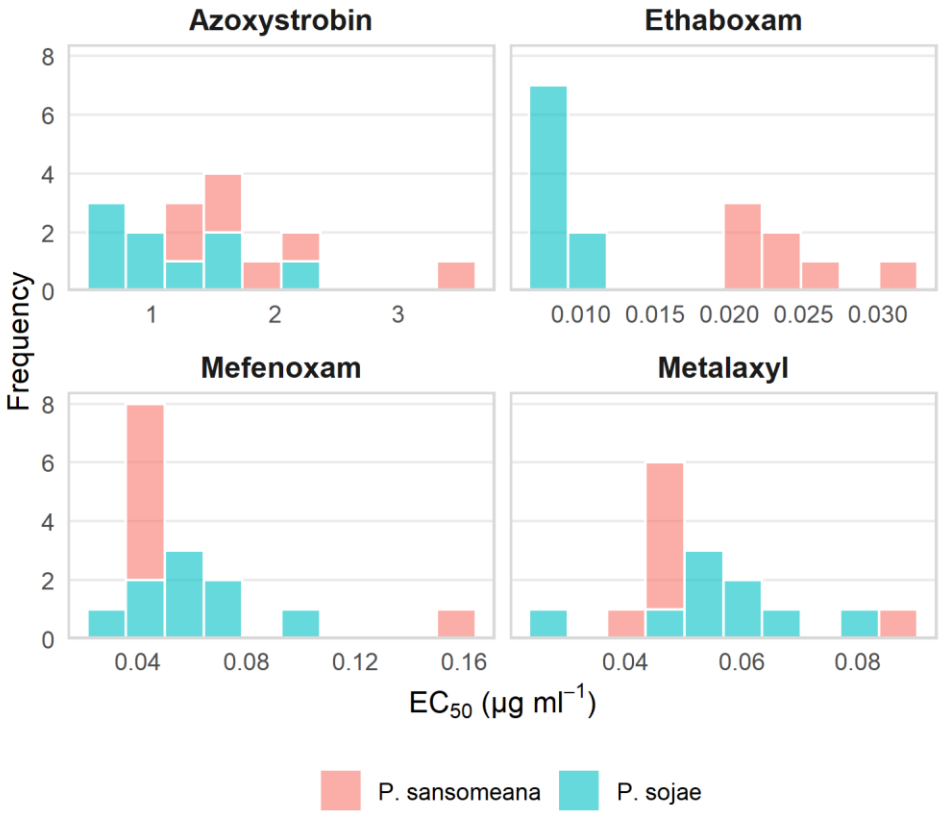

Figure 10. Distribution of EC_{50} values of *Phytophthora sansomeana* and *Phytophthora sojae* for their sensitivity to technical grade azoxystrobin, ethaboxam, mefenoxam and metalxyl.

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CHAPTER 3: AGGRESSIVENESS AND FUNGICIDE SENSITIVITY OF PYTHIUM SPECIES ASSOCIATED WITH ILLINOIS SOYBEAN FIELDS

ABSTRACT

Seedling diseases are caused by multiple *Pythium* species. *Pyhtium* spp. are managed using seed treatments, but sensitivity can vary by species. *Pythium* spp. was isolated between 2016 and 2017 through soil baiting from 26 soybean fields. Ten species were identified from 62 isolates recovered from 17 counties. *Pythium ultimum* var. *ultimun* was the most abundant species (40%). A seed plate assay was used to assess the aggressiveness of isolates on soybean and corn seeds. All species reduced germination of soybean seeds relative to the non-inoculated control, but only *Pythium ultimum* var. *ultimun* and *Py*. *aphanidermatum* reduced germination of corn seeds. Fungicide sensitivity of 11 isolates was assess using poison plate assays. All isolates were sensitive to metalaxyl and mefenoxam, but 27% and 18% of the isolates were insensitive to ethaboxam and azoxystrobin, respectively.

INTRODUCTION

Seedling diseases, one the most destructive diseases of soybean in Illinois, have caused losses of approximately 872 million US dollars in the state in the past two decades (Bandara et al. 2020). Seedling diseases are caused by a complex of species including those from *Pythium, Fusarium* and *Rhizoctonia solani* (Broders et al., 2007; Rojas et al., 2017a). The genus *Pythium* is commonly associated to this disease since more than 40 virulent species to soybean have been reported across the US Midwest (Rojas et al. 2017a). Disease symptoms include pre- and post-damping off as well as yellowing and stunting in seedlings (Schroeder et al. 2013). Symptoms

are very similar to those caused by other oomycete species, so it is difficult to determine which species are present in fields. Regional populations can vary across states and fields, so characterization of local populations is needed for more precise management recommendations (Rojas et al. 2017b).

In Illinois, multiple species have been isolated from soybeans including virulent and non-virulent species (Jiang et al. 2012; Rojas et al. 2017a). Jiang et al. (2012) identified 27 species from 12 fields distributed in six counties, 37% were virulent on soybean seedlings. *Pythium oopapillium, Pythium diclinum* and *Pythium irregulare* were the most abundant. Similarly, Rojas et al. (2017) found 30 species in 12 fields across two years (2011-2012) and 43% were virulent on soybean. In this study, *Pythium sylvaticum, Py. irregulare* and *Py. oopapillum* were the most abundant. In another study, (Noel et al. 2020) reported 17 species from two fields across two years (2016-2017) and the most abundant species were *Pythium heterothallicum, Py. irregulare* and *Py. sylvaticum*. Although species composition varied across studies, *Py. sylvaticum, Py. irregulare, Pythium ultimum* var. *ultimun, Pythium ultimum* var. *sporangiiferum* and *Pythium torolosum* were consistently recovered across the three studies. These species have also been consistently isolated across other states and are reported as virulent to soybeans (Broders et al. 2007a; Zitnick-Anderson & Nelson 2015; Radmer et al. 2017; Rojas et al. 2017). All these studies have reported high diversity in the state, but results are limited to few fields and counties.

No resistant cultivars are commercially available, so management of this disease relies mainly on seed treatment (Schroeder et al. 2013; Scott et al. 2020). In addition, cultural practices such as no-tillage, crop rotation and early planting can increase disease development (Broders et al. 2007a). Soils under no-tillage and early planting are normally wet and cold which can favor *Pythium* presence (Kirkpatrick et al. 2006). Soybeans are normally rotated with corn, but crop

rotation is not an effective management tool since multiple species are virulent to both crops (Radmer et al. 2017; Rojas et al. 2019). Seed treatment combinations of fungicides with different active ingredients is recommend since efficacy can vary depending on the species and fungicide (Noel et al. 2019). A combination of ethaboxam and metalxyl or mefenoxam has been proven the best option for large range of *Pythium* spp. (Rojas et al. 2019; Scott et al. 2020).

No recent data on *Pythium* diversity, aggressiveness, and fungicide sensitivity is available for Illinois. This information is needed to improve disease management and overall understanding the impact of *Pythium* infections in the state. The objectives of this study are to characterize the aggressiveness of *Pythium* spp. isolates and evaluate sensitivity to fungicides commonly used in seed treatments.

MATERIALS AND METHODS

Isolates

Pythium isolates were recovered in 2016 and 2017 from 26 fields across 17 counties. All isolates were recovered from soil baiting and identified by sequencing the ITS region using ITS 4/ITS 5 primers as described in chapter 2. A total of 63 isolates from 10 species were identified: *Py. ultimum* var. *ultimum* (*n* = 40), *Py. aphanidermatum* (*n* = 4), *Py. pleroticum* (*n* = 4), *P. yorkensis* (*n* = 4), *Py. torolosum* (*n* = 2), *Py. acanthophoron* (*n* = 2), *Py. ultimum* var. *sporangiiferum* (n = 2), *Py. vexans* (*n* = 2), *Py. acrogynum* (*n* = 1) and *Py. irregulare* (*n* = 1).

Soybean and corn seed assay

A seed plate assay was used to evaluate aggressiveness of all 63 *Pythium* isolates on soybean seed. Isolates were grown at room temperature (21°C) in water agar (500 ml distilled water, 10 g of agar) for three days. Nine mm plugs from each isolate were transferred to a plate

(100 x 15 mm) with water agar. Eight surface sterilized soybean seeds of the cultivar Pioneer 93Y25 were placed around the plug. Seeds were approximately 10 mm from the edge of the plate. Seeds were surface sterilized for 1 min in 70% ethanol, 1 min sodium hypochlorite and then rinsed in sterilized water. Seeds were dried in the laminar flow for 30 minutes before transferring them to plates. Plates were incubated at room temperature in shelfs with a light bank set for a 12-hour photoperiod. Controls consisted of plates with a PDA plug. In addition, one *Py. irregulare* and one *Py. ultimum* var. *ultimum* were included as positive controls. These species were selected because they have been identified as virulent in multiple studies. The experiment was a completely randomized design with three replicates (plate) for each isolate and was run twice. The number of germinated and colonized seeds were counted after five and 10 days.
In addition, the seed plate assay described above was used to evaluate aggressiveness on corn seed. Twenty isolates from six species were selected: *Py. ultimum* var. *ultimum* (*n* = 13), *P. aphanidermatum* (*n* = 2), *Py. torolosum* (*n* = 2), *Py. pleroticum* (*n* = 1), *Py. ultimum* var. *sporangiiferum* (*n* = 1) and *Py. vexans* (*n* = 2). The 108-day hybrid Munson was used in the assay. The experiment was the same as the soybean seed assay with the exception that the *Py. irregulare* control was not included and plates were rated after five days only.

Soybean seedling assay

A cup assay was used to evaluate the aggressiveness of three Pythium isolates on soybean seedlings. The only *Py. irregulare* isolate and one randomly selected *Py. ultimum* var. *ultimum* isolate were used in this assay. These two isolates were compared against a *Pythium sylvaticum* isolate recovered in IL in another survey. Styrofoam cups were filled up to the half with a soil mix (1:1:1, soil:peat:perlite) and mixed with either 10 or 20 ml of colonized millet. Then, four soybean seeds (Pioneer 93Y25) were placed on top of soil and covered with an extra

50 ml of soil mix. Cups were placed in a growth chamber at 23°C and a 12-hour photoperiod. Controls consisted of 10 ml of millet, 20 ml millet, and soil only. The experiment was a completely randomized design with three replicates (cups) for each species and the whole experiment was conducted two times. Number of germinated seedlings was counted after 10 days. Seedlings were removed from cups and soil was removed with tap water. Seedlings were placed in paper envelopes and dried at 50°C in a laboratory oven. Seedlings dry weight was measured after 24 hours.

Fungicide sensitivity

Poison plate assays were used to assess the sensitivity of 11 *Pythium* isolates to azoxystrobin, ethaboxam, mefenoxam, and metalaxyl. Same protocol used in chapter 2 to evaluate sensitivity of *Phytophthora* spp. isolates was used. The concentrations used were 0, 0.1, 0.5, 0.1, 1 and 10 µg/ml for azoxystrobin, mefenoxam and metalaxyl; 0, 0.1, 1, 5, 10, 50 and 100 µg/ml for ethaboxam. For azoxystrobin, 25 mg of salicylhydroxamic acid (SHAM) (Fisher Scientific) was added to the media to avoid the alternative oxidase pathway (Broders et al. 2007).

Data analysis

Analysis was performed in R version 4.0.3 (R Core Team 2020). A generalized linear model was fit to the count data from seed assays with species, isolates and experiment as fixed factors. No effect of experiment was observed so it was removed from the models. A linear model was fit to data from seedling assays with species, millet and experiment as fixed factors. No effect of millet and experiment was observed so it was removed from the models. Mean separations were conducted using Tukey's HSD at $\alpha < 0.05$. The effective fungicide concentration to reduce the growth by 50% (EC_{50}) was calculated using the best fitting model (LL.3) in the R package “drc” (Ritz et al. 2015).

RESULTS

Aggressiveness to soybean

Aggressiveness on soybean seeds varied by species and time it was measured. Overall, *Py. ultimum* var. *ultimum*, *Py. ultimum* var. *sporangiiferum* and *Py. aphanidermatum* were the most aggressive species (Figure 11). Significant differences between species were observed for germination and number of colonized seeds at both five and 10 days ($P < 0.001$). For number of colonized seeds at five days, only *Py. ultimum* var. *ultimum*, *Py. ultimum* var. *sporangiiferum* and *Py. aphanidermatum* were significantly different from the control. On average, these three species have colonized 33% of the seeds at day five compared to none for the rest of isolates.

At 10 days, for both colonized seeds and germination, all the species were significantly different from the control (Figure 11; Figure 12). Although all the species were significantly different from the control at 10 days, the three most aggressive species were significantly different from the rest of the species except *Py. irregulare*. No seed inoculated with *Py. ultimum* var. *ultimum*, *Py. ultimum* var. *sporangiiferum* , *Py. aphanidermatum* and *Py. irregulare* germinated and on average 94% of the seeds were colonized. On average, 25% of the seeds inoculated with *Py. vexans*, *Py. acanthophoron*, *Py. yorkensis* and *Py. torolosum* germinated and 63% were colonized. *Pythium acrogynum* the less aggressive species (Figure 12). Fifty percent of the seeds inoculated with *Py. acrogynum* germinated and 43% were colonized. None of the seeds in PDA control were colonized and 70% germinated.

Isolate was a significant factor for germination and colonized seeds at 5 and 10 days ($P < 0.001$). For colonized seeds at five days, differences between isolates from the same species were observed for *Py. pleroticum*, *Py. ultimum* var. *ultimum* and *Py. ultimum* var. *sporangiiferum*. For germination and colonized seeds at 10 days, differences between isolates

were observed for *Py*. *pleroticum*, *Py*. *torolosum* and *Py*. *yorkensis*. Differences between isolates of *Py*. *yorkensis* were observed for germination.

Aggressiveness to corn

Aggressiveness on corn seeds varied by species for germination and colonized seeds at five days ($P < 0.001$). From the six species tested, only *Py*. *ultimum* var. *ultimum* and *Py*. *aphanidermatum* were significantly different from the control for germination and colonized seeds (Figure 13). Although not significantly different from the control, *Py*. *torulosum* and *Py*. *ultimum* var. *sporangiiferum* were not significantly different from *Py*. *ultimum* var. *ultimum* and *Py*. *aphanidermatum*.

Soybean seedling assay

No difference between 10 ml and 20 ml of inoculum was observed, so it was removed from the model and data was combined. Significant differences between the control none and the control millet were observed, so species were compared against the control millet. Three species significantly reduced seedling emergence and weight ($P < 0.001$). *Pythium irregulare* was the most aggressive species followed by *Py*. *ultimum* var. *ultimum* and *Py*. *sylvaticum* (Figure 14). *Pythium irregulare* was significantly different from *Py*. *sylvaticum* for emergence and weight and from *Py*. *ultimum* var. *ultimum* for weight only. No difference between *Py*. *ultimum* var. ultimum and *Py*. *sylvaticum* was observed for either emergence or weight.

Fungicide sensitivity

Sensitivity to fungicides varied by species and fungicide (Figure 15). *Pythium aphanidermatum* and *Py*. *vexans* were insensitive to ethaboxam and azoxystrobin, respectively. Relative growth was higher than 50% at the higher concentration for these two species, so no

EC_{50} values for these fungicides was calculated. On average, mefenoxam was the most effective fungicide (0.83 µg/ml) followed by azoxystrobin (0.98 µg/ml), metalaxyl (1.10 µg/ml) and ethaboxam (20.9 µg/ml). Less sensitive species were observed for all fungicides (Fig. 15). *Pythium torolosum* was the least sensitive species to ethaboxam, mefenoxam and metalaxyl. *Pythium acanthophoron*, *Py*. *irregulare*, *Py*. *vexans* and *Py*. *ultimum* var. *sporangiiferum* had EC_{50} values < 3.5 µg/ml. In contrast, *Py*. *torolosum* values for ethaboxam ranged from 10 – 65 µg/ml. For mefenoxam and metalaxyl, all species were sensitive to concentrations < 0.6 µg/ml while values for *Py*. *torolosum* ranged from 1 – 4 µg/ml for these fungicides. For azoxystrobin, *Py*. *ultimum* var. *sporangiiferum* and *Py*. *irregulare* were less sensitive compared to the rest of species that an EC_{50} value was calculated.

DISCUSSION

Multiple *Pythium* species cause seedling diseases. Diagnosis in the field can be difficult since symptoms are identical for all *Pythium* species and very similar for other oomycetes like *Phytophthora* spp. *Pythium* populations can vary between states as well as their aggressiveness and fungicide sensitivity, so management recommendations should be targeted to a specific state or region (Rojas et al. 2017b ; Matthiesen & Robertson, 2021). The objective of this study was to characterize aggressiveness and fungicide sensitivity of *Pythium* isolates recovered from soybean fields in Illinois. Ten species were identified from 25 fields in 17 counties. All species were able to colonize and reduce germination of soybean seeds, but only two species were virulent on corn seeds. Differences in aggressiveness and fungicide sensitivity were observed among species, but little variation among isolates from same species was observed.

Pythium ultimun var. *ultimun* was the most abundant species across Illinois fields. Rojas et al. (2017a) found this species in 10 of the 11 US states surveyed, where most of soybean production is concentrated. In this same study, 84 oomycete species were identified from which *Py. ultimun* var. *ultimun* was the sixth and fourth most abundant species across two years, respectively. Temperature affects both abundance and aggressiveness of many oomycetes, but not for *Py. ultimun* var. *ultimun* (Matthiesen et al. 2016; Rojas et al. 2017a; Navarro-Acevedo et al. 2021). This species abundance is not affected by temperature and has been found to cause disease at both 15°C and 25°C (Navarro-Acevedo et al. 2021). It also one of the most aggressive species across different temperatures (13 - 20°C) (Jiang et al. 2012; Radmer et al. 2017; Rojas et al. 2017a). In our study, *Py. ultimun* var. *ultimun* was highly aggressive in both seed and seedling assays. Noel et al. (2019) found that *Py. ultimun* var. *ultimun* was the most abundant species in high disease pressure environments in Michigan. In this study, most of the fields sampled had seedling disease history and it was recovered from half of the counties sampled and in 36% of the fields.

All species were able to colonize and reduce germination of soybean seeds, but aggressiveness varied by species. Our results agree with results from other studies that have found that *Py. ultimum* var. *ultimum*, *Py. ultimum* var. *sporangiiferum* and *Py. irregulare* are among the most aggressive species on soybean (Zitnick-Anderson & Nelson, 2015; Coffua et al. 2016; Radmer et al. 2017; Rojas et al. 2017a). *Pythium aphanidermatum* was also one of the most aggressive species in this study. Rojas et al. (2017a) reported this species as virulent to soybean in both seed and seedling assay. In contrast, Jiang et al. (2012) reported that *Py. aphanidermatum* isolates recovered from Illinois were not virulent to soybean in a seed plate assay.

Although significantly different from the control, *Py. pleroticum*, *Py. vexans*, *Py. acanthophoron*, *Py. yorkensis*, *Py. torolosum* and *Py. acrogynum* were less aggressive on soybean seeds. All these species have been reported as non-virulent or as weak pathogens of soybean (Jiang et al. 2012; Coffua et al. 2016; Radmer et al. 2017; Rojas et al. 2017a; Veterano et al. 2018). *Pythium pleroticum* and *Py. acanthophoron* have been reported as non-virulent in all studies (Jiang et al. 2012; Coffua et al. 2016; Radmer et al. 2017; Rojas et al. 2017a). All these studies have used the same seed plate assay used in our study, but a rating scale instead of count data. This could explain why they were designated as non-virulent compared to our study. Possibly under a rating scale these species would have been designated as non-virulent in our study since they colonized few seeds and cause little germination reduction. *Pythium torolosum*, *Py. acrogynum* and *Py. vexans* have been classified as non-virulent or virulent depending on the study. Jiang et al. (2012) and Coffua et al. (2016) reported *Py. torolosum* isolates from Illinois and Pennsylvania as non-virulent. In contrast, isolates from Ohio caused low to moderate disease in soybean (Dorrance et al. 2004; Broders et al. 2007a). Mathiessen and Robertson (2021) reported that *Py. torolosum* was more aggressive at 13°C and that isolates from different states varied for aggressiveness. Isolates from Illinois were among the least aggressive at this temperature. Jiang et al. (2012) reported that *Py. acrogynum* isolates recovered from Illinois were not virulent on soybean in seed plate assays at 22°C. In contrast, Radmer et al. (2017) reported that *Py. acrogynum* isolates from Minnesota were virulent at 25°C, but not at 20°C or 15°C.

Only *Py. ultimum* var. *ultimum* and *Py. aphanidermatum* were able to cause disease in both soybean and corn. Isolates baited from soils using soybean seedlings are commonly more aggressive to soybean compared to corn (Broders et al. 2007; Radmer et al. 2017). Our study

agrees with other studies that *P. ultimum* var. *ultimum* is an aggressive pathogen of both soybean and corn (Broders et al. 2007; Coffua et al. 2016; Radmer et al. 2017). *Pythium ultimum* var. *sporangiiferum* and *Py. irregulare* have also reported as pathogenic to corn, but in this study, they were not significantly different from the control (Radmer et al. 2017; Rojas et al. 2019). *Pythium pleroticum* and *Py. acrogynum* were not virulent in corn in our study and both have been reported as non-virulent in other studies (Coffua et al. 2016; Radmer et al. 2017; Rojas et al. 2019). Radmer et al. (2017) reported that corn seedlings inoculated with *Py. pleroticum* had higher root mass compared to the control. *Pythium torolosum* was not significantly different from the control in our study. Coffua et al. (2016) reported *Py. torolosum* isolates recovered from soybean as non-virulent on corn at 25°C. In contrast, Rojas et al. (2019) reported that *P. torolosum* isolates recovered from corn seedlings were virulent on corn at 20°C, but not at 13°C.

Sensitivity of *Pythium* spp. varied depending on the fungicide and the species. Overall, mefenoxam and metalaxyl were the most effective fungicides. All the species were sensitive to these fungicides at < 4 µg/ml which agrees with other studies that *Pythium* is sensitive to these fungicides (Noel et al. 2019; Rojas et al. 2019; Noel et al. 2020). Both fungicides have the same active ingredient and have low risk of developing resistance as seed treatment (Noel et al. 2019; FRAC 2020). In contrast, azoxystrobin has a high risk of resistance and insensitive *Pythium* isolates have been reported (Broders et al. 2007; Radmer et al. 2017; FRAC 2020). Azoxystrobin is broad spectrum fungicide included in seed treatments to control *Pythium*, *Fusarium* and *Rhizoctonia solani*. In this study, *Py. vexans* was insensitive to the higher concentration used in this study (10 µg/ml). This is the first report of azoxystrobin insensitive strains of *Py. vexans*. *Pythium aphanidermatum* was considered insensitive to ethaboxam in this study. Ethaboxam at 100 µg/ml did not inhibit growth of this species. *Pythium aphanidermatum* has been previously

reported to have inherent resistance to ethaboxam (Noel et al. 2019). No species was insensitive to all fungicides, but *Py*. *torolosum* was less sensitive to all fungicides compared to sensitive species.

FIGURES

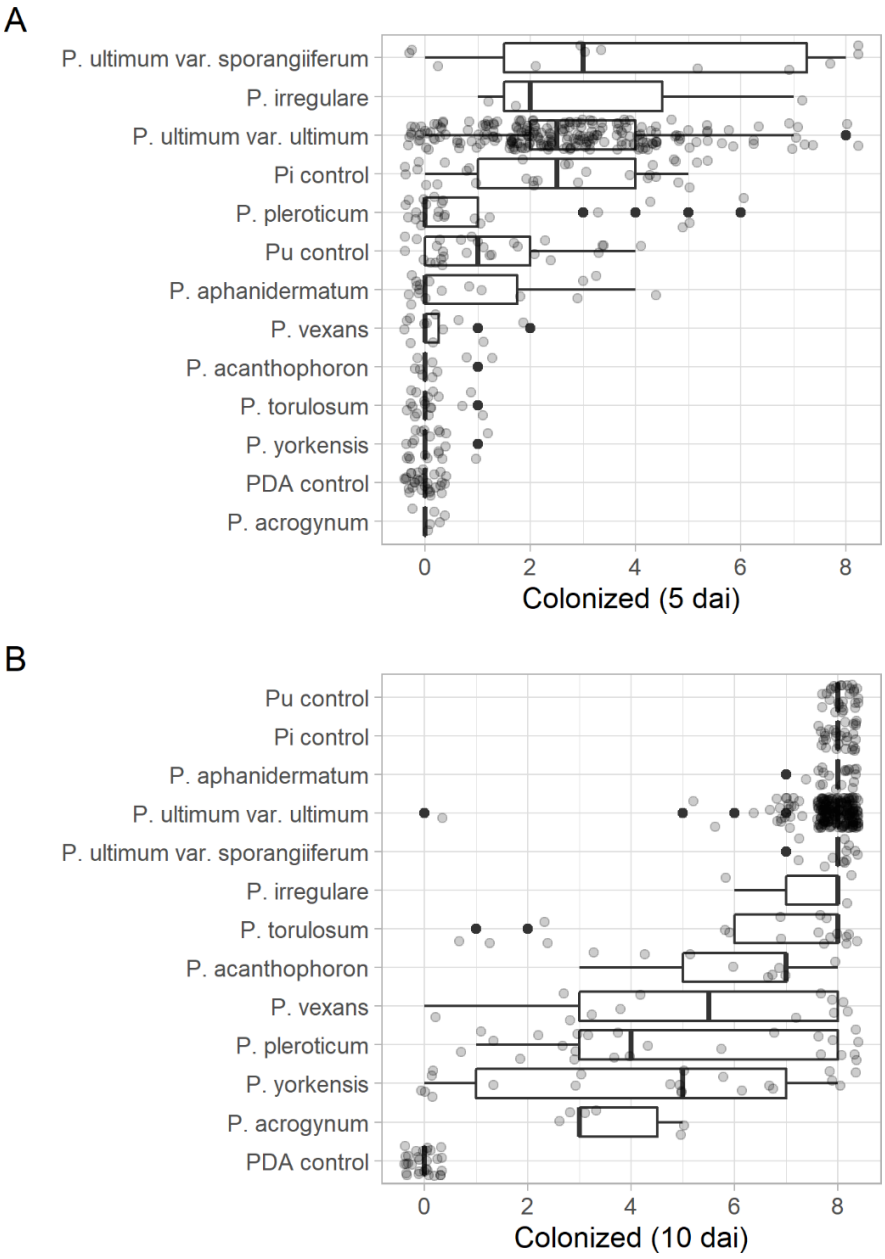

Figure 11. Distribution of number of colonized (A) and germinated (B) corn seeds at day five after inoculation with *Pythium* spp. Center lines represent the medians and black solid dots represent outliers. Box limits indicate the 25th and 75th percentiles and whiskers extend 1.5 times the interquartile range.

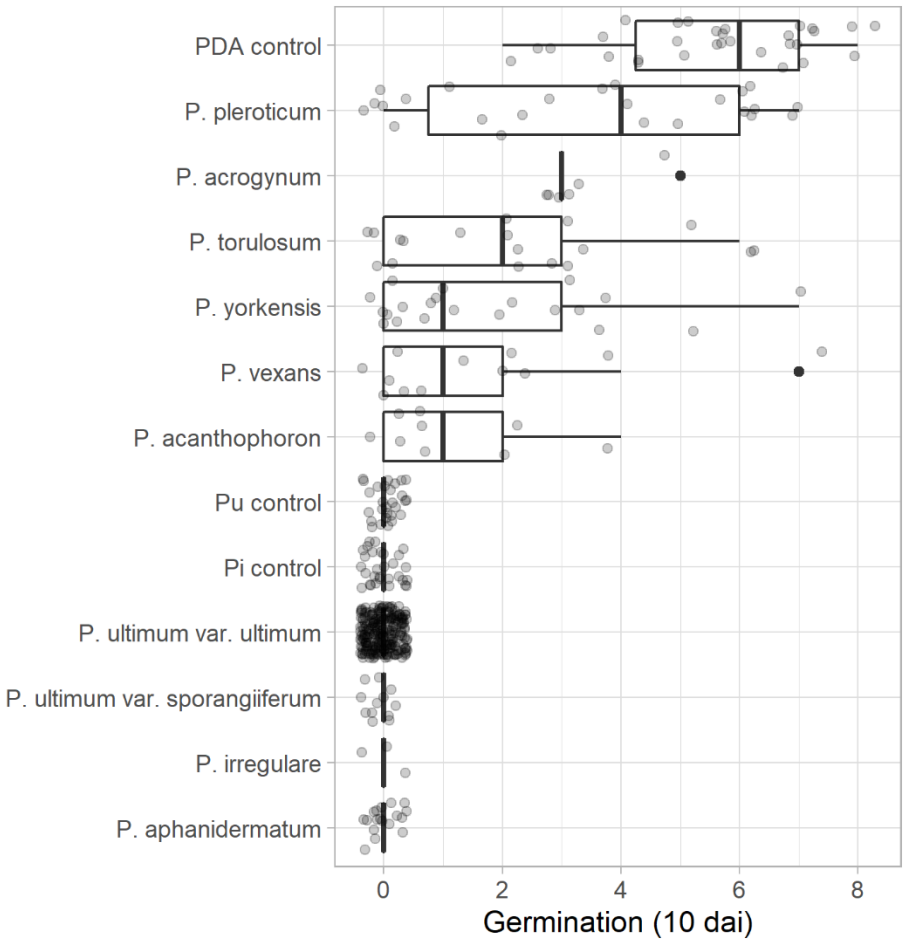

Figure 12. Distribution of germinated soybean seeds at day 10 after inoculation with *Pythium* spp. Center lines represent the medians and black solid dots represent outliers. Box limits indicate the 25th and 75th percentiles and whiskers extend 1.5 times the interquartile range.

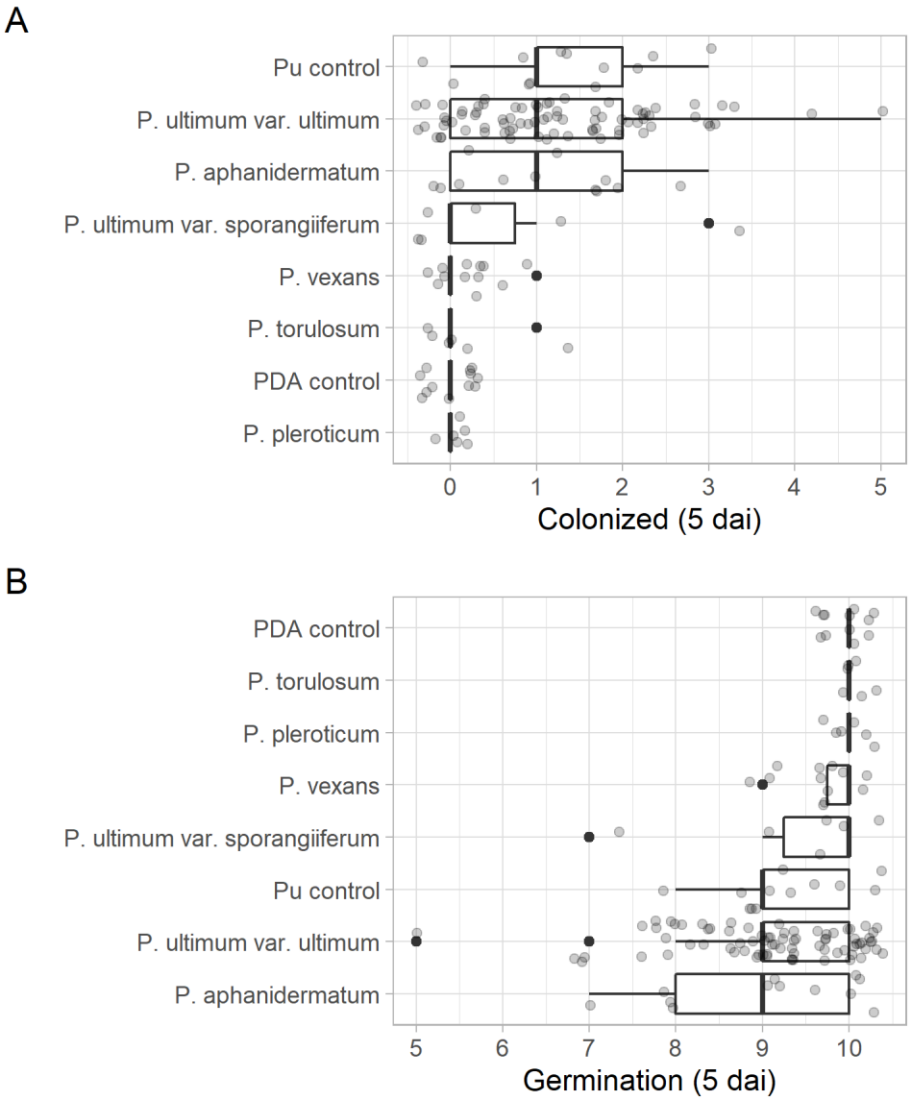

Figure 13. Distribution of number of colonized (A) and germinated (B) corn seeds at day five after inoculation with *Pythium* spp. Center lines represent the medians and black solid dots represent outliers. Box limits indicate the 25th and 75th percentiles and whiskers extend 1.5 times the interquartile range.

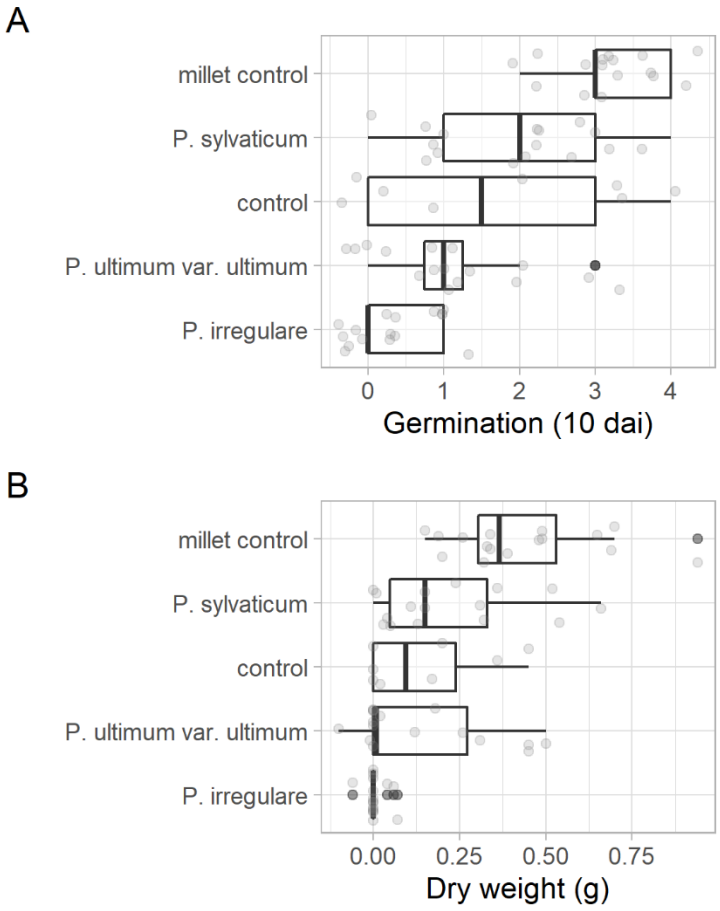

Figure 14. Distribution of number of germinated soybean seedlings (A) and dry weigh of seedlings (B) after inoculation with colonized millet with *Pythium* spp. Center lines represent the medians and black solid dots represent outliers. Box limits indicate the 25th and 75th percentiles and whiskers extend 1.5 times the interquartile range.

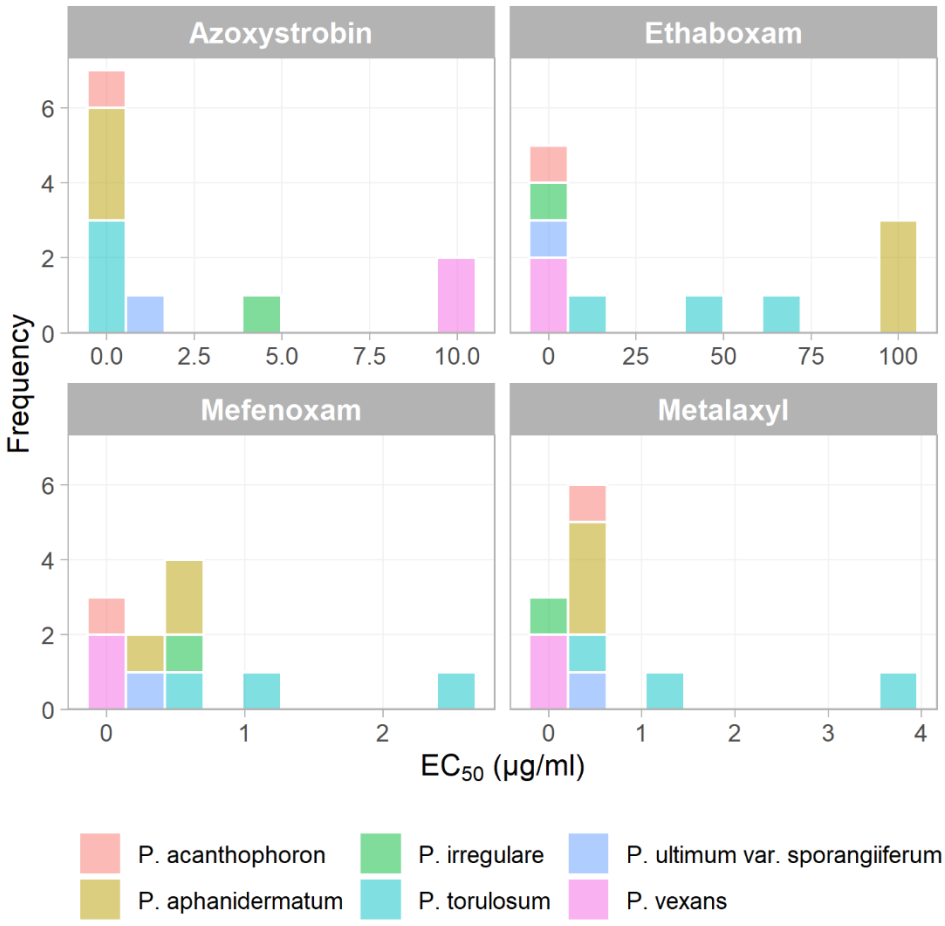

Figure 15. Distribution of EC_{50} values of *Pythium* species for their sensitivity to technical grade azoxystrobin, ethaboxam, mefenoxam and metalxyl.

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